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Cardiovascular Effects of LAS 30538, a New Vascular Selective Ca^{2+}-Channel Blocker

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Abstract—A new compound, 1-[2-(2,6-dimethylphenoxy)ethyl]-α,α-bis-(*p*-fluorphenyl)-4-piperidine methanol (LAS 30538), was found to have potent vasodilator effects. Its vasorelaxant activity was demonstrated in rat perfused hindlimbs contracted with 80 mM K^+, having an IC50 value of 40 nM. In conscious spontaneously hypertensive rats, LAS 30538 administered orally, caused dose-dependent sustained falls in systolic blood pressure with an ED30 value of 11 mg kg^{-1}. In pithed rats, LAS 30538, strongly inhibited vasoconstriction induced by the α_2-adrenoceptor agonist B-HT 933 and the calcium agonist compound Bay K8644 with ED50 values of 4 mg kg^{-1} p.o. and 1·3 mg kg^{-1} i.v., respectively. Results from electrophysiological studies carried out using guinea-pig papillary muscles partially depolarized by 22 mM K^+ are consistent with LAS 30538 acting as a Ca^{2+}-channel blocker. When compared with verapamil, in guinea-pig and rabbit isolated heart preparations, LAS 30538 caused less cardiodepression and bradycardia. The results suggest that LAS 30538 may have some advantages over other Ca^{2+}-channel blockers such as verapamil in causing less myocardial depression for a given level of vasodilatation.

Ca^{2+}-Channel blockers, by their properties of inhibiting influx of Ca^{2+} across cell membranes (Fleckenstein 1977), have been one of the major therapeutic advances for the treatment of cardiovascular disorders, including hypertension and angina (Ellrodt et al 1980).

In our laboratory, we have run a specific programme to identify novel Ca^{2+}-channel blockers displaying vascular vs cardiac selectivity, in order to avoid cardiodepression. We now describe the cardiovascular effects of LAS 30538, (1-[2-(2,6-dimethylphenoxy)ethyl]-α,α-bis-(*p*-fluorphenyl)-4-piperidine methanol (Fig. 1), a novel halogenated diphenylpiperidine derivative. This compound has been previously classified by us as a Ca^{2+}-entry blocker (Bou et al 1991).

The vasodilator, antihypertensive and cardiac effects of LAS 30538 were compared with those of three well known Ca^{2+}-entry blockers, verapamil, diltiazem and flunarizine (Henry 1980; Spedding 1985). In addition, electrophysiological studies were carried out with LAS 30538 in order to confirm its mechanism of action as a Ca^{2+}-channel blocker.

Materials and Methods

Effects on vascular smooth muscle

Rat perfused hindlimbs. Male Wistar rats (Almirall), 300–350 g, were heparinized (1000 int. units kg^{-1}, i.p.) and killed by a blow to the head. The abdominal aorta was cannulated to allow a perfusion of the hindlimbs with Tyrode solution (for composition of solutions see 'Solutions and drugs' section) at 4·5 mL min^{-1} by means of a peristaltic pump (Watson-Marlow). The temperature of the perfusate was maintained at 37°C throughout the experiment. To register perfusion pressure, a transducer (Letica, TRA-021) was situated between the animal and the peristaltic pump. With a constant flow rate of 4·5 mL min^{-1}, perfusion pressure was maintained at values close to 25 mm Hg and maximal values of 150 mm Hg were achieved in preliminary experiments by application of high concentrations of KCl (K^+ 80 mM). The method is a modification of that described by Korstanje et al (1988). Briefly, after stabilizing for 20 min with Tyrode solution two or three vasoconstrictions were elicited by perfusion with high potassium Tyrode solution. Tyrode solution was then changed to a solution containing verapamil, diltiazem, flunarizine or LAS 30538 at concentrations of 1, 10 and 100 nM and following equilibration the solution was changed to one containing K^+ 80 mM as well as the drug under test. This operation was repeated 10 and 20 min after the beginning of treatment. Responses were calculated as percentage inhibition of contraction.

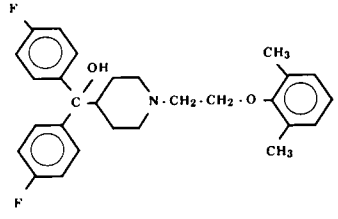

FIG. 1. Structural formula of LAS 30538.

Rat isolated aorta. Aortae from male Wistar rats were placed in oxygenated Krebs solution at room temperature (21°C), cleaned of connective tissue, and cut into rings of 2 mm length. Each aortic ring was suspended in a 30 mL organ bath filled with Krebs solution continuously gassed with 5% CO_2 in O_2 at 37°C. The aortic rings were attached by a cotton thread to an isometric force transducer (Letica TRI-010) coupled to a pen recorder (Letigraph 4000) to monitor force generation. Preparations were subjected to a resting force of 1 g and were allowed to equilibrate for at least 60 min. The

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bath K^+ concentration was increased by 60 mM to induce contraction both before and after treatment with LAS 30538, verapamil or flunarizine (0·01–10 μM). Results were calculated as the percentage inhibition of basal contraction.

Conscious spontaneously hypertensive rats (SHR). Thirteen-week old male SHR (Charles River, Spain) were kept for 3 weeks in standard conditions of temperature, humidity and lighting, with free access to food and water. During this time, the animals were trained to remain calm at a temperature of 37°C in restraining holders to allow indirect measurement of systolic blood pressure by means of a tail cuff (Letica 5000). For details, see Llenas (1985). After several basal blood pressure measurements, verapamil, diltiazem, flunarizine or LAS 30538 were given orally at doses of 3, 10 and 30 mg kg^{-1} and blood pressure was measured again 1 h later.

Effects on myocardial contractility

Rabbit isolated papillary muscles. Male rabbits (Prolabor), 2·5–3 kg, were stunned by a blow to the head and their hearts quickly removed and placed in a dissection bath containing oxygenated Krebs solution at room temperature (21°C). The papillary muscles from the right ventricle were carefully dissected from the heart, suspended in a 30 mL organ bath containing Ringer-Locke solution, and stimulated electrically (1 ms, 1 Hz and supramaximal voltage). The solution was gassed with O_2 at 37 ± 0·1°C. The isometric tension developed by the preparation was measured with a force transducer (Letica TRI-010) and recorded on a pen recorder (Letigraph 4000) through a suitable preamplifier (Letica PRS 205). Drugs were administered using a cumulative concentration procedure.

Guinea-pig isolated working hearts. Preparations were established essentially according to Flynn et al (1978). In brief, male heparinized (1000 int. units kg^{-1}, i.p.) guinea-pigs (Dunkin-Hartley, Charles River) weighing 400–450 g, were killed by a blow to the head. Hearts were rapidly excised and perfused through the aorta at 50 cm H_2O pressure with an oxygenated (5% CO_2 in O_2) Krebs solution maintained at 37 ± 0·1°C.

The hearts were then prepared for the working mode by inserting a glass cannula into the left atrium. After 20 min equilibration, the hearts were switched into the working mode, using a left atrial filling pressure of 12 cm H_2O and an afterload of 73 cm H_2O. Left ventricular dP/dt_{max} was measured as an index of contractility (Flynn et al 1978) before and after administration of 0·1, 0·3 or 1 μM LAS 30538, verapamil or flunarizine using a cumulative concentration procedure. Results were expressed as percentage changes.

Mechanism of action studies

Electrophysiological studies on guinea-pig isolated papillary muscles. Male guinea-pigs (Dunkin-Hartley, Charles River), 400–450 g, were stunned by a blow to the head and after a midsternal thoracotomy, the hearts were quickly excised and placed in oxygenated Krebs solution. Papillary muscles (2 mm wide) from the right ventricle were tied by the tendons with a fine silk suture, and then cut proximally to the tie. The distal connection of the muscle in the ventricular wall was cut and then tied to an isometric transducer (HSE-F30). The preparations were placed horizontally in a plexiglass tissue bath of 3 mL volume, superfused (6 mL min^{-1}) with Krebs solution at 37 ± 0·1°C, gassed with 5% CO_2 in O_2.

Electrical stimulation (Grass S88—Stimulator) was made by a bipolar platinum electrode (1 ms, 0·5 Hz and supramaximal voltage). A resting tension of 0·5–1 g was applied yielding the maximal contractile force and isometric tension recorded.

Transmembrane action potentials (APs) were recorded through glass electrodes (10–20 M ohm resistance) filled with 3 M KCl connected to a high-impedance unity gain electrometer.

The first derivative of AP with respect to the time (dV/dt_{max}) was obtained by an HSE differentiator (model 664). Data were acquired and processed with a Cibertec analogue-digital converter (Physiorec-3) and recorded on a Panasonic stereo video tape recorder (model NV-FS1).

In another series of experiments, slow response action potentials were induced in the papillary muscles. After the exposure to a high-K^+ (22 mM) solution, which was prepared by isotonic substitution of NaCl with KCl, slow action potentials were evoked by the application of supramaximal electrical stimuli in the presence of histamine 1 μM (Inui & Imamura 1976). Preparations were allowed to equilibrate for at least 2 h before the start of the experiments. The concentration of LAS 30538 in the superfusion medium was increased stepwise at intervals of 20 min. Only results from experiments in which a stable response was maintained throughout are presented.

Effects on vasopressor responses to B-HT 933 and Bay K8644 in pithed rats. Male Wistar rats (Almirall), 250–300 g, were anaesthetized with sodium pentobarbitone (70 mg kg^{-1}, i.p.), respired with room air through a tracheal cannula connected to a respiratory pump (Harvard model 680), and pithed as described by Spedding (1982). The left carotid and jugular vessels were cannulated to measure blood pressure and administer the agonists, respectively. One hour before pithing, animals received, p.o., verapamil, diltiazem, flunarizine, LAS 30538 or vehicle. In the experiments using Bay K8644 as agonist, verapamil, flunarizine or LAS 30538 were administered by the i.v. route 15 min beforehand.

The effect of drugs on pressor responses induced by the selective α_2-adrenoceptor agonist B-HT 933 (1 mg kg^{-1}, i.v.), or by the Ca^{2+} agonist Bay K8644 (30 μg kg^{-1}, i.v.) were measured.

Effects on cardioaccelerator responses in pithed rats. Male Wistar rats (Almirall), 300–350 g, were anaesthetized with pentobarbitone sodium (60 mg kg^{-1}, i.p.). After tracheal cannulation, the rats were pithed with a stainless steel rod and immediately respired (45 strokes min^{-1}; 2 mL 100 g body weight) via a Harvard small animal respirator pump (model 680). A jugular vein was cannulated for administration of drugs. Arterial blood pressure was recorded from a carotid artery using a Letica transducer (Letica TRA-021). Heart rate was derived from the phasic arterial pressure signal with a ratemeter (Letica CAR 1000). Both parameters were displayed on a Letigraph 4000 recorder. The pithing rod was

positioned such that the tip lay in the thoracic portion of the spinal cord. All rats received (+)-tubocurarine (1·5 mg kg^{-1}, i.v.) and were bilaterally vagotomized.

The cardioaccelerator response was obtained by continuous electrical stimulation (1 ms, 0·5 Hz and supramaximal voltage) of the thoracic spinal cord using the pithing rod as the stimulating electrode, an indifferent electrode being placed in the back region. Rats exhibiting a tachycardia of less than 75 beats min^{-1} were rejected from these experiments.

When the cardioaccelerator response had stabilized, LAS 30538 at 0·3 mg kg^{-1} (i.v.) was administered. Five minutes later, calcium gluconate (1 mg min^{-1}, i.v.) or saline (0·1 mL min^{-1}) was infused over 40 min (Braun ED Secura infusion pump).

Statistical analysis

Statistical comparisons were made using Student's *t*-test, assuming significance at $P<0·05$.

Solutions and drugs

LAS 30538 was prepared by the Chemistry Department of Almirall Laboratories. Diltiazem, verapamil and flunarizine were obtained from Impex Química (Mollet del Vallés, Spain), calcium gluconate from Aldrich Chemie (Steinheim, Germany) and (+)-tubocurarine from Gayoso Wellcome (Alcalá de Henares, Spain). B-HT 933 and Bay K8644 were obtained as gifts from Dr Karl Thomae (Biberach, Germany) and Bayer AG (Wupperthal, Germany), respectively.

For in-vitro experiments or for i.v. administration, drugs were dissolved in 20% polyethylene glycol 300 in distilled water and diluted with physiological saline where necessary. When compounds were administered by the oral route they were suspended in methylcellulose 0·5% and Tween 80 0·1% (dose volume 10 mL kg^{-1}).

The compositions of the physiological solutions were as follows: Tyrode solution (mM): NaCl 130, KCl 5·6, $CaCl_2$ 2·2, $MgCl_2$ 0·6, $NaHCO_3$ 24·2, NaH_2PO_4 1·2 and glucose 11; high-potassium Tyrode solution (mM): NaCl 58, KCl 80, $CaCl_2$ 3·6, $MgCl_2$ 2, $NaHCO_3$ 12, NaH_2PO_4 0·8 and glucose 6; Krebs solution (mM): NaCl 118, KCl 4·7, $CaCl_2$ 2·5, $MgSO_4$ 1·6, NaH_2PO_4 1·2, $NaHCO_3$ 25 and glucose 11; Ringer–Locke solution (mM): NaCl 157·4, KCl 5·6, $CaCl_2$ 2·1, $NaHCO_3$ 1·8 and glucose 5·6.

Results

Relaxation of rat perfused hindlimbs and aortic rings

LAS 30538 (1–100 nM) caused a concentration-dependent inhibition of the vasoconstriction induced by high-potassium in rat perfused hindlimbs (Fig. 2). Over the same concentration range, verapamil and diltiazem were less potent than LAS 30538 but similar in potency to flunarizine (Fig. 2).

LAS 30538 with a threshold concentration of 10 nM caused concentration-related inhibition of K^+-induced contractions in the rat isolated aorta. Verapamil had a similar potency, whereas flunarizine was less active in this test (Fig. 3).

Antihypertensive effects in the conscious SHR

LAS 30538 produced dose-dependent falls in systolic blood pressure measured 1 h after oral administration (Fig. 4). The

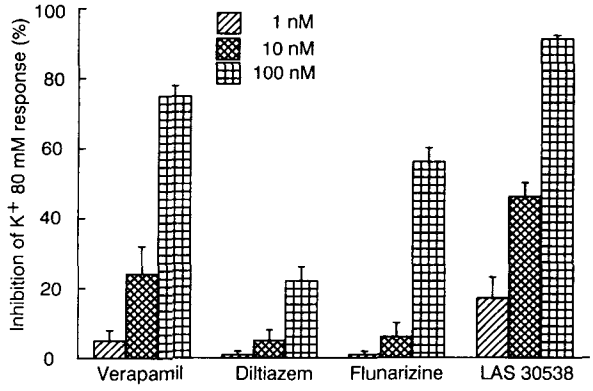

FIG. 2. Comparative effects of verapamil, diltiazem, flunarizine and LAS 30538 on the high-potassium (80 mM) responses in the rat perfused hindlimbs. Results are expressed as means ± s.e.m. (n = 8).

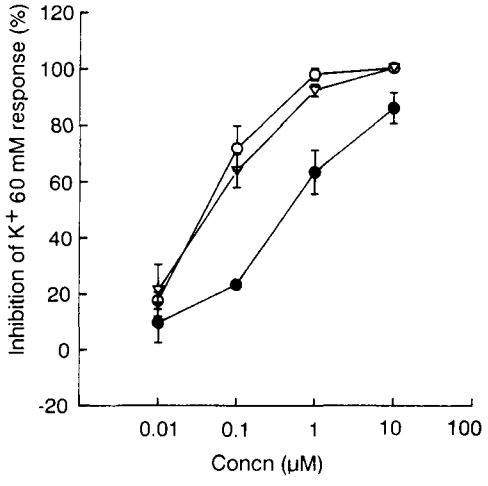

FIG. 3. Comparative effects of verapamil (○), flunarizine (●) and LAS 30538 (▽) on the rat aortic rings in response to K^+ 60 mM. Results are expressed as means ± s.e.m. (n = 4).

effects of verapamil in lowering blood pressure were similar to those of LAS 30538. The effects of diltiazem and flunarizine were modest, achieving 14 and 22% decreases, respectively, in blood pressure at 30 mg kg^{-1} (Fig. 4).

The effects of the drugs on heart rate were relatively minor (less than 30% increase) over the range of doses administered.

Effects on guinea-pig and rabbit cardiac preparations

On isolated papillary muscles and in the working heart preparation, LAS 30538 (0·1–1 μM) produced negative inotropic effects at concentrations between 10 and 30 times higher than did verapamil, whilst diltiazem and flunarizine had a similar negative inotropic potency to LAS 30538 (Figs 5, 6).

Electrophysiological studies on guinea-pig papillary muscles

The effects of LAS 30538 at concentrations of 10 and 30 μM on force of contraction and AP parameters of ventricular preparations are shown in Table 1. Force of contraction was diminished gradually following cumulative addition of LAS 30538 and effects on the AP duration at 50% repolarization

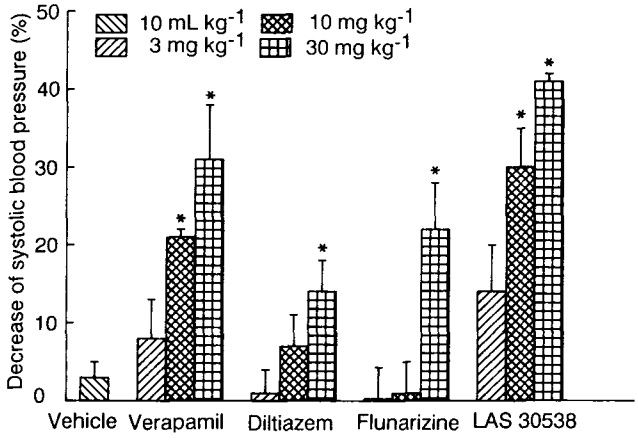

FIG. 4. Comparative effects of vehicle, verapamil, diltiazem, flunarizine and LAS 30538 administered orally on systolic blood pressure in the spontaneously hypertensive rat. Results are expressed as means ± s.e.m. (n = 6). *$P < 0.05$ vs vehicle treated group.

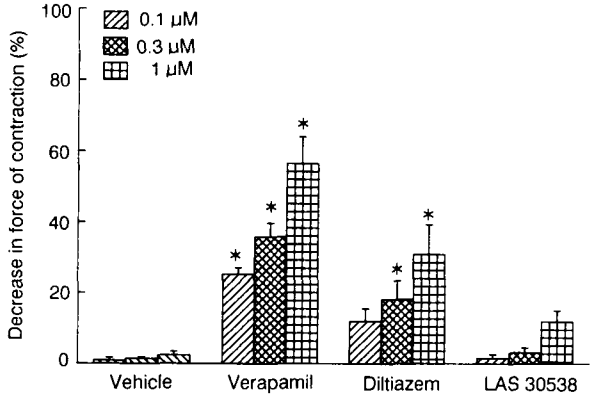

FIG. 5. Comparative effects of verapamil, diltiazem and LAS 30538 on the rabbit papillary muscle preparation. Results are expressed as means ± s.e.m. (n = 4). *$P < 0.05$ vs vehicle treated group.

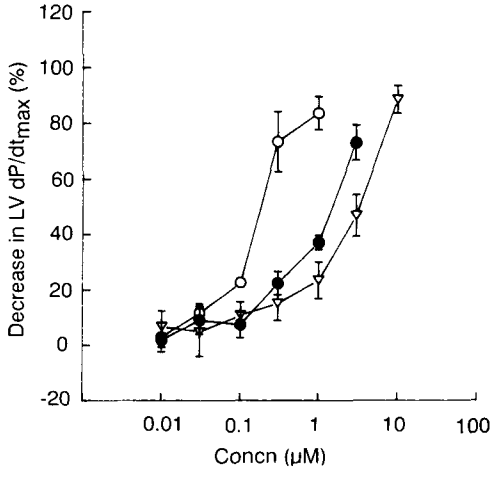

FIG. 6. Comparative effects of verapamil (○), flunarizine (●) and LAS 30538 (▽) on the left ventricular dP/dt_{max} (LV dP/dt_{max}) in the guinea-pig isolated working heart preparation. Results are expressed as means ± s.e.m. (n = 5).

(APD50) was also decreased by up to 21 ± 6·2% of the control values. In contrast, dV/dt_{max} was practically unaffected. Steady-state effects of each concentration were reached within 20 min.

On the partially depolarized preparations, where the action potential configuration is very dependent on calcium entry, LAS 30538 up to 10 μM produced decreases in force of contraction, APD50 and dV/dt_{max} (Table 2).

Inhibition of pressor responses in pithed rats

Pressor responses induced by Bay K8644 in pithed rats were inhibited dose-dependently by LAS 30538 and to a lesser extent by verapamil. Flunarizine in contrast was ineffective (Fig. 7a).

B-HT 933 pressor responses were inhibited by all drugs tested, LAS 30538 being the most potent (Fig. 7b).

Reversion of the inhibitory effect on the cardioaccelerator responses by calcium gluconate

The effects of calcium gluconate (1 mg min^{-1}) had little or no effect on either basal heart rate or electrically stimulated tachycardia (see Clapham 1988). As is shown in Fig. 8, LAS 30538 (0·3 mg kg^{-1}) caused inhibition of the cardioaccelerator response that was markedly reversed after 40 min infusion of 1 mg kg^{-1} calcium gluconate.

Discussion

The present studies show that LAS 30538 has potent vasodilator activity. Thus, LAS 30538 was found to inhibit K^+ 60 and 80 mM induced contractions on the rat aorta and rat hindlimbs, respectively. The potency of this action was greater in the rat perfused hindlimbs than that in rat aorta suggesting that LAS 30538 has more specificity for resistance than conductance vessels. The effects of LAS 30538 after oral administration on blood pressure were observed in the spontaneously hypertensive rat, where doses of 10 and 30 mg kg^{-1} orally decreased systolic blood pressure (30 and 41%, respectively) without important effects on heart rate (< 15% change).

Parallel experiments performed on isolated cardiac preparations demonstrated that at the same concentrations that produce smooth muscle relaxation in-vitro, LAS 30538 had little effect on contractility of the guinea-pig and rabbit isolated hearts. Over the same concentration range verapamil was shown to have clear cardiodepressant activity. These results indicate that LAS 30538 has a much higher vascular vs cardiac selectivity compared with verapamil. Diltiazem similarly exhibited a vascular vs cardiac selectivity although it appeared to be less selective than LAS 30538.

The experiments carried out with pithed rats showed that LAS 30538 inhibits the vasopressor responses induced by the selective α_2-adrenoceptor agonist B-HT 933 and by the calcium agonist Bay K8644 which act by opening the receptor- and voltage-operated Ca^{2+}-channels, respectively (Kanmura et al 1984; Yamamoto et al 1984; Haeusler et al 1986; Timmermans et al 1987).

Previous studies using [^{3}H]nitrendipine have demonstrated that LAS 30538 has affinity (K_i = 236 nM) for the dihydropyridine binding sites of rat cortex (Bou et al 1991), a result consistent with Ca^{2+}-channel blocking activity.

Table 1. Effects of LAS 30538 on action potential parameters and force of contraction in guinea-pig papillary muscles driven at 0·5 Hz.

	APD90 (ms)	APD50 (ms)	dV/dt_{max} ($V\ s^{-1}$)	Force of contraction (mN)
Basal values	160±7	133±6	173±24	0·88±0·5
LAS 30538 (μM)				
10 (n=9)	−4·1±0·9*	−6·4±1·6*	−1·1±2	−13·5±4
30 (n=6)	−17·8±4·5*	−21·0±6·2*	−7·5±4	−29·5±9*

Values are % of change (mean±s.e.m.). APD90 and APD50, action potential duration at 90% and 50% of repolarization, respectively. *$P<0·05$ compared with basal values.

Table 2. Effects of LAS 30538 on slow action potential parameters and force of contraction in guinea-pig papillary muscles driven at 0·5 Hz.

	APD50 (ms)	Force of contraction (mN)	dV/dt_{max} ($V\ s^{-1}$)
Basal values	138±4	0·80±0·08	11·4±4
LAS 30538 (μM)			
0·1	1·3±1·3	−8·1±7·2	−8·8±2·5*
1	−8·7±2·6*	−21·1±9·4*	−10·8±8·8
10	−14·5±6·0*	−38·9±6·3*	−18·3±5·9*

Values are % of change (mean±s.e.m., n=4). APD50, action potential duration at 50% of repolarization. *$P<0·05$ compared with basal values.

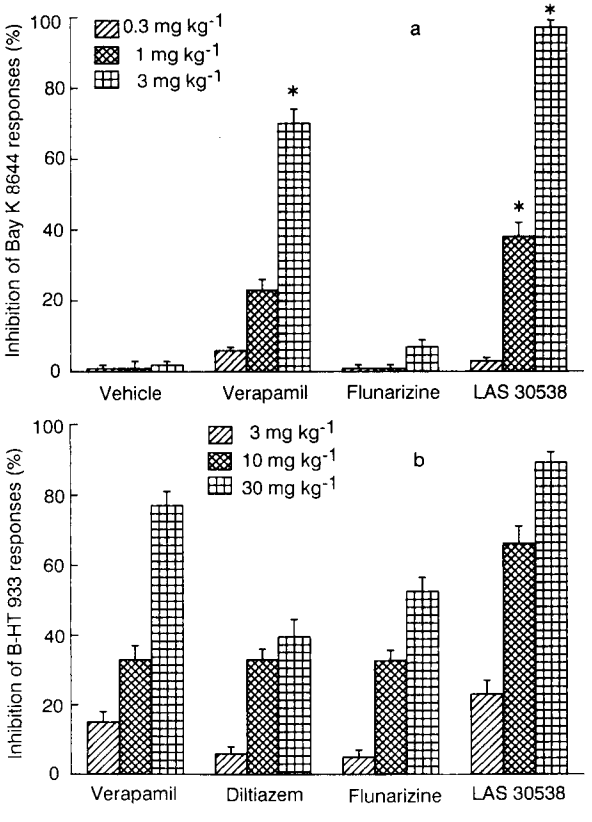

FIG. 7.a. Comparative effects of verapamil, flunarizine and LAS 30538 administered i.v. upon Bay K8644 vasopressor responses in pithed rats. Results are expressed as means±s.e.m. (n=5). *$P<0·05$ vs vehicle treated group. b. Comparative effects of verapamil, diltiazem, flunarizine and LAS 30538 administered orally upon B-HT 933 vasopressor responses in pithed rats. Results are expressed as means±s.e.m. (n=5).

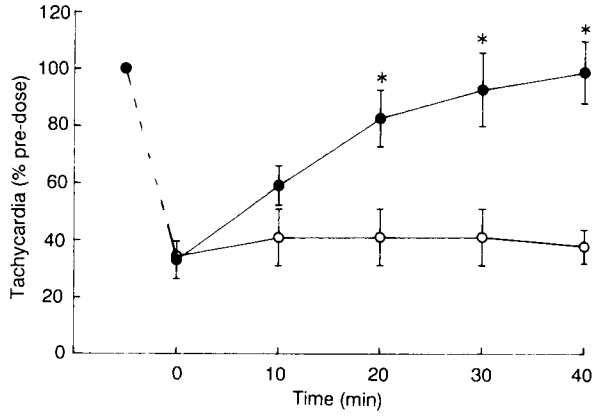

FIG. 8. Effects of calcium gluconate on the inhibition by LAS 30538 i.v. of the electrically-evoked tachycardia in the pithed rat preparation. The heart rate before drug administration was taken as 100%. Values are expressed as mean±s.e.m. (n=5). *$P<0·05$ vs saline-infused group. LAS 30538+vehicle ○, LAS 30538+Ca^{2+}-gluconate ●.

Further evidence of blockade of calcium entry by LAS 30538 was obtained in the present studies. Thus, electrophysiological studies carried out in guinea-pig papillary muscles demonstrated that LAS 30538, at concentrations 10 to 30 times higher than those that produced vascular relaxation, inhibited the slow action potentials in guinea-pig papillary muscles depolarized by 22 mM K^+. Although the V_{max} of slow action potentials is an indirect estimate of the Ca^{2+} current (Schneider & Sperelakis 1975), these results are consistent with LAS 30538 having Ca^{2+}-channel blocking properties. A comparison of effects on force of contraction in partially depolarized preparations and normal preparations indicated that the negative inotropic effects of LAS 30538 are voltage-dependent. Similar findings have been obtained with other Ca^{2+}-channel blockers such as diltiazem (Lee & Tsien 1983) and verapamil (Ehara & Kaufmann 1978; Gristwood et al 1985). Additional evidence for Ca^{2+}-channel blockade was shown by the reversal of the LAS 30538 inhibition of the cardioaccelerator response to thoracic stimulation of sympathetic nerves in pithed rats following infusion of calcium gluconate (for rationale see Clapham (1988)). Haeusler (1972) found that the Ca^{2+}-channel blocker verapamil had no effect on [^{3}H]noradrenaline release in the cat isolated heart. Thus, it is likely that the mechanism of the inhibition of sustained tachycardia by LAS 30538 occurs postsynaptically at the pacemaker cells of the sinoatrial and atrioventricular nodes. These nodes, lacking a fast inward sodium current, rely on current carried by calcium (Katz 1983).

In conclusion, the present studies indicate that LAS 30538 is a Ca^{2+}-channel blocker exhibiting selectivity for the vasculature with little cardiac depressant action. The above data suggest that LAS 30538 could be useful in the treatment of hypertension. However, we have also presented data (Gristwood et al 1992) which show that LAS 30538 is an extremely potent compound at inhibiting insulin secretion and producing hyperglycaemia in-vivo, which could represent an undesirable side-effect for vasodilator therapy.

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