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Selective cardiodepressant activity of fluodipine, a fluorenone-1,4-dihydropyridine derivative

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Abstract

The effect of the dihydropyridine derivative, 1,4-dihydro-2,6-dimethyl-4-(fluorenon-4-yl)pyridine-3,5-dicarboxylic acid diallyl ester (fluodipine) was studied in vitro in different rabbit, rat and guinea pig preparations and in vivo in the rabbit in order to characterize its pharmacological profile at cardiac and at vascular sites. Compared to nifedipine, fluodipine showed a similar cardiodepressant activity, and a much lower inhibitory activity on vascular contraction. The highest tissue selectivity was observed in guinea pig preparations: fluodipine was about 2–3 times more effective than nifedipine on chronotropism and inotropism in isolated atria, and about 150 times less effective on aortic strip contraction. Accordingly, fluodipine (i) showed high-affinity binding to guinea pig ventricular L-type cardiac Ca^{2+} channels ($K_i = 2.57$ nM), (ii) was about 80 times less effective than nifedipine to inhibit Ca^{2+} influx in vascular smooth muscle cells and (iii) induced a significant reduction of heart rate in the anesthetized rabbit ($ID_{25} = 8.5$ mg kg^{-1}, i.v.) without affecting the blood pressure up to 20 mg kg^{-1}, whereas nifedipine showed a significant hypotensive effect at very low doses ($ID_{25} = 0.18$ mg kg^{-1}, i.v.). The pacemaker current I_f of rabbit sino-atrial node myocytes was not affected by fluodipine. These findings demonstrate that fluodipine exerts selective cardiodepressant activity, likely due to a higher affinity for cardiac than for vascular Ca^{2+} channels.

Keywords: Ca^{2+} channel antagonist; Fluorenone-1,4-dihydropyridine derivative; Cardiac/vascular species specific selectivity, in vitro; Bradycardic activity

1. Introduction

Heart rate is a major determinant of myocardial energy demand (Braunwald, 1976). Thus, drug-induced bradycardia should be beneficial in ischemic heart disease, because it reduces myocardial oxygen consumption and increases blood flow to the subendocardial layers of the myocardium which are predominantly perfused during diastole (Buckberg et al., 1975; Friedman et al., 1975; Indolfi and Ross, 1993). The pharmacological agents that lower heart rate include Ca^{2+} channel blockers (such as verapamil, diltiazem and 1,4-dihydropyridine derivatives) and β-adrenoceptor antagonists. All these drugs also exert other cardiac and vascular effects that either complement or antagonize the bradycardiac action, and extra-cardiovascular, often undesirable, effects.

A reduction in myocardial oxygen consumption, resulting from decreased heart rate and contractility, contributes to the anti-ischemic effects of currently available Ca^{2+} channels antagonists. However, particularly for 1,4-dihydropyridines, these effects may be almost entirely offset by baroflex sympathetic activation, due to the strong vasodilatation induced by these drugs. Moreover, some consequences of vasodilatation, such as headache and peripheral edema, are poorly tolerated side-effects (Habib and Roberts, 1994). It has been demonstrated that the replacement of the *o*-nitrophenyl ring of nifedipine (see Fig. 1A) with a tricyclic group determines potent and selective

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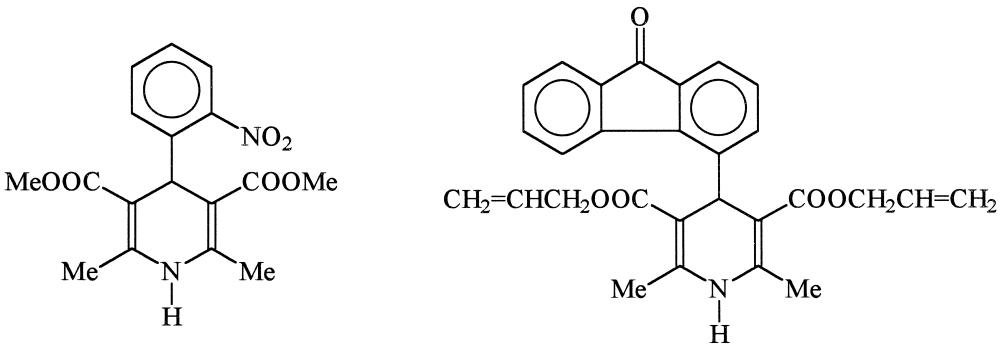

Fig. 1. Chemical structure of nifedipine (A) and fluodipine (B).

bradycardic effects (Valenti et al., 1990; Rampa et al., 1991). More recently it has been shown that xanthone and fluorenone-1,4-dihydropyridine derivatives with different 3,5 and 2 substituents of the 1,4-dihydropyridine nucleus (Rampa et al., 1991; Chiarini et al., 1992; Rampa et al., 1992; Bisi et al., 1993; Rampa et al., 1995; Budriesi et al., 1996), as well as xanthone derivatives bearing a chlorine atom on the tricyclic group (Bisi et al., 1996) also exert a selective bradycardic action. Of all 1,4-dihydropyridine derivatives mentioned above, the 1,4-dihydro-2,6-dimethyl-4-(fluorenon-4-yl)pyridine-3,5-dicarboxylic acid diallyl ester (fluodipine) appeared the most potent bradycardiac agent on in vitro cardiac preparations from guinea pigs (Rampa et al., 1992). In the present work we studied the effects of fluodipine (Fig. 1B), on various in vitro cardic and vascular preparations derived from guinea pigs, rats and rabbits with the aim of better characterizing its pharmacological profile. Fluodipine was also tested in vivo in the rabbit, in order to verify a potential therapeutic advantage over established pharmacological interventions. The effects of nifedipine were also studied in most preparations, thus allowing a direct comparison with the effects of the new 1,4-dihydropyridine derivative.

2. Materials and methods

2.1. Animals

Male Hartley guinea pigs (300–400 g), male Sprague–Dawley and Wistar rats (250–300 g) and male and female New Zealand rabbits (2.3–3.0 kg) obtained from Charles River (Calco, Como, Italy) were used. The animals were kept in air-conditioned rooms (21 ± 2°C and 60 ± 10% relative humidity) under a constant 12-h light:12-h dark cycle and received a standard diet and tap water. All procedures followed the guidelines of the animal care and use committee of the University of Bologna (Bologna, Italy).

2.2. In vitro experiments

2.2.1. Evaluation of chronotropic and inotropic activity in guinea pig, rat and rabbit left and right atria

Guinea pigs and Wistar rats were killed by cervical dislocation under i.m. ketamine (250 mg kg^{-1}) anaesthesia. Rabbits (2.5–3.0 kg) were killed by exsanguination under pentobarbital sodium anaesthesia (60 mg kg^{-1}, i.v.). The hearts were rapidly removed, washed by perfusion through the aorta with oxygenated physiological salt solution of the following composition (mM): 136.9 NaCl; 5.4 KCl; 2.5 $CaCl_2$; 1.0 $MgCl_2$; 0.4 $NaH_2PO_4 \times H_2O$; 11.9 $NaHCO_3$; 5.5 glucose and bubbled with 5% CO_2/95%O_2 at pH 7.4. The temperature was maintained at 35°C. Two isolated heart preparations were set up: spontaneously beating right atria and left atria driven at 1 Hz. Stimulation was carried out with rectangular pulses of 0.6–0.8 ms duration and 50% above threshold voltage through two platinum contact electrodes in the lower holding clamp (Grass S88 stimulator, Grass). Contractile activity was recorded isometrically by means of a force transducer (FT.03 Grass Instruments, Quincy, MA, USA) connected to a pen recorder (KV 380 Rangoni, Bologna, Italy). After the tissue had been beating for several minutes, a length-tension curve was obtained and the muscle was stretched to the length at which 90% of maximal force was developed. A stabilization period of 45–60 min was allowed before the atria were challenged with various agents. During the equilibration period, the bathing solution was changed every 15 min and the threshold stimulation voltage for the left atria was checked carefully. Atrial preparations were used to examine the inotropic and chronotropic activity of the compounds (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.5, 10, 50 μM) first dissolved in dimethylformammide, then diluted with the physiological salt solution described above. Dose–response curves were obtained by cumulative addition of the compounds. The concentration of compound in the organ bath was increased approximately 5-fold at each step, with each addition being made only after the response to the previous one had reached a

maximal level and remained steady. With this procedure, the concentration of dimethylformammide in the bath solution never exceeded 0.3%, a concentration which did not produce any appreciable inotropic or chronotropic effect.

2.2.2. Evaluation of inhibition of vascular contraction in guinea pig, rat and rabbit aortic strips

The thoracic aorta was removed and placed in Tyrode solution of the following composition (mM): 118 NaCl; 4.75 KCl; 2.54 $CaCl_2$; 1.20 $MgSO_4$; 1.19 KH_2PO_4; 25 $NaHCO_3$; 11 glucose. The vessel was cleaned of extraneous connective tissue. Two helicoidal strips (10 mm × 3 mm) were cut from each aorta beginning from the end proximal to the heart. Vascular strips were then tied with surgical thread (6-0) and suspended in a jacketed tissue bath containing physiological salt solution at 35°C and aerated with 5% CO_2/95% O_2 at pH 7.4. The strips were secured at one end to Plexiglass hooks and connected via surgical thread to a force-displacement transducer for monitoring changes in isometric contraction. Aortic strips were subjected to a resting force of 1 g (guinea pig and rat) or 1.5 g (rabbit) and washed every 20 min with fresh physiological salt solution for 1 h. After an equilibration period of 1 h, the aortic strips were contracted by washing in physiological salt solution containing 80 mM KCl (equimolar substitution of K^+ for Na^+). Subsequent to the contraction reaching a plateau (approximately 30 min) the compounds (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100 μM) were added cumulatively to the bath, allowing for any relaxation, to obtain an equilibrium level of force. Addition of the drug vehicle had no appreciable effect on the K^+-induced level of force (dimethylformammide for all compounds).

2.2.3. Evaluation of pacemaker current I_f modulation in rabbit sino-atrial node myocytes

Myocytes from the sino-atrial node region of female rabbits were isolated following a procedure previously described (Di Francesco et al., 1986). Spindle shaped, spontaneously active myocytes were superfused with normal Tyrode solution containing (mM): 140 NaCl; 5.4 KCl; 2 $CaCl_2$; 1 $MgCl_2$; 5.5 D-glucose; 5 HEPES–NaOH; adjusted to pH 7.4, at a temperature of 36.5°C. Currents were measured by means of patch clamp recording in the whole-cell configuration (Axopatch 200-A, Axon Instr., CA, USA), with pipettes having a tip resistance of 3–5 MΩ. Series resistance (R_s) were compensated to 85%–90% of their initial value (5–15 MΩ). The pipette solution contained (mM): 10 NaCl; 130 aspartic acid; 146 KOH; 2 $MgCl_2$; 2 ATP-Na salt; 5 creatine phosphate; 0.1 GTP; 5 EGTA; 2 $CaCl_2$ (calculated free $Ca^{2+} = 10^{-7}$ M); 10 HEPES–KOH; pH 7.2. The I_f current was measured as the time-dependent component activated by 600 ms hyperpolarizing steps from a holding potential (E_h) of −35 mV. A step to potentials positive to E_h (e.g., −5 mV) followed hyperpolarization to allow for complete I_f deactivation between subsequent pulse protocols. During I_f measurements 1 mM $BaCl_2$ and 2 mM $MnCl_2$ was added to Tyrode solution to minimize contamination by K^+ and Ca^{2+} currents, respectively. Data were recorded on a PCM video recording system during the experiment then analyzed digitally off-line by replaying them into a 12-bit A/D board (sampling rate 2 kHz). Collagenase was obtained from Worthington Biochemical (Freehold, NJ, USA); elastase and protease from Sigma (Milano, Italy).

2.2.4. Evaluation of Ca^{2+} influx inhibition in rat aortic smooth muscle A_7r_5 cells

The Ca^{2+} channel blocking activity was evaluated in A_7r_5 cells (aortic smooth muscle cells obtained from Sprague–Dawley rat embryos) (Kimes and Brandt, 1976) loaded with fura-2 acetoxymethyl ester (AM) as described in detail previously (Spampinato et al., 1993). The cell line was obtained from the American Type Culture Collection (Bethesda, MD, USA). Cells were grown in 75-cm^2 plastic culture flasks at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 1 μM biotin and 1 μM lipoic acid in a humidified atmosphere under 5% CO_2/95% air. Confluent cell layers were detached by treatment with 0.05% trypsin (Gibco). Cells were harvested by gentle agitation in warm solution A, containing (in mM) 137 NaCl, 5.4 KCl, 0.17 Na_2HPO_4, 0.22 KH_2PO_4, 5 glucose and 58 sucrose, resuspended (4×10^6 cells ml^{-1}) in solution B, containing 130 NaCl, 5 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 20 HEPES, pH 7.4, with an additional 0.5 mg ml^{-1} bovine serum albumin (solution C). The cell suspension was then incubated with 2 μM fura-2 AM for 20 min in a shaking water bath at 37°C. The cells were pelleted by low-speed centrifugation, resuspended in solution B with an additional 5 mg ml^{-1} bovine serum albumin and incubated for another 10 min at 37°C. The cells were then centrifuged, resuspended (2×10^6 cells ml^{-1}) in solution C and kept at room temperature. Fluorescence was measured with a Perkin-Elmer LS-5 fluorescence spectrophotometer at excitation and emission wavelengths of 340 and 510 nm, 5 and 10 nm slit width, respectively. The cells (about 2×10^6 cells/2 ml per cuvette) were kept in suspension at 37°C with a magnetic stirrer. Depolarization was induced by adding 150 mM KCl to the cell suspension. The change in fluorescence (ΔF) was calculated from the difference between final (determined 3 min after addition of KCl) and initial (determined 30 s prior KCl) values. The effect of the compounds assayed on the K^+-induced increase of cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ was established by preincubating the fura-2 AM-loaded cells with vehicle or with different concentrations of drugs for 3 min before the challenge with KCl. The IC_{50} values, defined as the concentration of drug giving a half-maximal decrease in ΔF, were calculated using logit analysis. Assuming a uniform distribution of intracellular Ca^{2+}, $[Ca^{2+}]_i$ was calculated as previously described (Spampinato et al., 1993), according to the

equation: $[Ca^{2+}]_i = K_d \ [(F_i - F_{min})/(F_{max} - F_i)]$, where K_d is the apparent dissociation constant of fura-2 for Ca^{2+} (225 nM), F_i is the fluorescence signal in arbitrary units of intact fura-2 AM-loaded cells, and F_{min} and F_{max} are the minimum and maximum fluorescence after addition of 5 mM EGTA + 30 mM Tris and 0.05% Triton X-100 + 6 mM $CaCl_2$, respectively. Background autofluorescence, measured in unloaded cells from the same preparation, was subtracted from all the measurements. Completeness of the intracellular hydrolysis of fura-2 AM was verified by recording the emission spectrum of fura-2 from 300 to 600 nm in the loaded cells: the fluorescence peak was only seen at 340 nm at the end of the loading procedure. Moreover, stimulation of the cells did not lead to any loss of intracellularly trapped fura-2, as ascertained by fluorescence measurements (data not shown).

2.2.5. Binding assays

Membranes were prepared according to the method of Glossmann and Ferry (1985), using guinea pig heart ventricles obtained from male Hartley guinea pigs. All steps of tissue preparation were performed at 4°C unless otherwise indicated. The tissues were finely minced and homogenized in 3 vol ice-cold buffer solution containing 20 mM $NaHCO_3$ and 0.1 nM phenylmethylsulphonylfluoride, pH 7.4 with a Polytron homogenizer. The homogenate was diluted 1:7 in the same buffer solution and centrifuged at $1500 \times g$ for 15 min. The supernatant was collected and centrifuged at $48\,000 \times g$ for 15 min; the pellet was washed twice by homogenization under the conditions already described in 7 vol ice-cold 50 mM Tris/HCl, 0.1 mM phenylmethylsulphonylfluoride buffer, pH 7.4, with centrifugation at $48\,000 \times g$ for 15 min. The final pellet was resuspended in 3 vol of the same buffer, and the membrane suspension was divided into aliquots and stored at −80°C. On the day of the experiment, aliquots of membranes were thawed, resuspended in 50 mM Tris/HCl, 1 mM $CaCl_2$ buffer, pH 7.4 and used for the binding assay (100 μg of protein/tube). Protein was determined by the method of Lowry et al. (1951).

Binding experiments were performed under conditions of near-darkness. Saturation binding experiments were performed at concentrations ranging from 0.05 to 5 nM of [^{3}H]isradipine (spec. activity 80 Ci mM^{-1}, obtained from NEN Life Science Products; $K_d = 4.3 \times 10^{-11}$). Specific binding was determined in the presence of 1 μM nifedipine. Ventricular membranes were incubated at 25°C for 120 min in a final volume of 1 ml and incubation was stopped by vacuum filtration over Whatman GF/C filters. Competition binding experiments were performed using 0.2 nM [^{3}H]isradipine. Drugs were prepared as stock solutions (10^{-2} M) in ethanol and protected from light. Drug dilutions were made into assay buffer. Bound radioactivity was measured in a Beckman Ls 1701 scintillation counter and the data were analyzed using the LIGAND program (Munson and Rodbard, 1980). At least three independent experiments with assays in duplicate were done for each compound.

2.3. In vivo experiments

2.3.1. Evaluation of chronotropic activity and arterial pressure modulation in the anesthetized rabbit

Male New Zealand white rabbits, weighing 2.3–2.8 kg were used for these studies. The rabbits were anesthetized and maintained with a continuous infusion of ketamine through a catheter inserted in the marginal vein of the ear (7 mg kg^{-1} followed by 0.8 mg kg^{-1} min^{-1}). Heart rate and arterial pressure were continuously recorded 10 min before and 10 min after each treatment with a catheter inserted in the central ear artery and connected to a Statham transducer. Heart rate was derived from a rate-meter triggered from the arterial pulse. In preliminary experiments, heart rate was also evaluated in conscious rabbits kept in comfortable polycarbonate restrainers for approximately 30 min before the start of the experiment. Test drugs were i.v. administered as a bolus and effective bradycardic doses were determined using cumulative doses ranging from 0.05 to 20 mg kg^{-1}. The dose was increased every 10 min and the heart rate was evaluated 5 min after each injection, the time at which maximal effect was achieved with the compound (as ascertained in preliminary experiments). The injection of vehicle alone did not modify the heart rate (see Table 4). In the anesthetized rabbits, treated with the vehicle alone, blood pressure and heart rate remained stable during the entire experimental period (data not shown). All drugs were dissolved in 5% (wt./vol) glucose solution containing 10% (vol/vol) ethanol and 1% (vol/vol) Tween 80 (vehicle) immediately before administration. The inhibitory dose, able to reduce by 25% (ID_{25}) heart rate or arterial pressure, was calculated by iterative curve-fitting, using the Inplot program (GraphPad Software, San Diego, CA). The changes in heart rate induced in vivo by the drugs were calculated as percentages of the control value (recorded immediately before drug administration) and are presented as means ± S.E.M. The significance of differences between means was evaluated by one-way analysis of variance (ANOVA) and Duncan's test.

2.4. Data analysis

Data from in vitro experiments were analyzed with Student's *t*-test. The criterion for significance was a P value of < 0.001. The concentrations needed to produce 30% and 50% inhibitory effects (EC_{50}, EC_{30} and IC_{50}) were calculated from log concentration–response curves (Probit analysis according to Litchfield and Wilcoxon, $n = 6$–8; Tallarida and Murray, 1991). All data are presented as means ± S.E.M.

Table 1
Chronotropic and inotropic activity on cardiac in vitro preparations

Species	% Decrease (mean ± S.E.M.)		EC_{30} on chronotropism		EC_{50} on chronotropism		EC_{50} on inotropism	
	Negative chronotropic activity[a] ($n = 7–9$) at 10^{-7} M	Negative inotropic activity[b] ($n = 7–8$) at 5×10^{-5} M	EC_{30}^{c} (μM)	95% Confidence limit (10^{-6})	EC_{50}^{c} (μM)	95% Confidence limit (10^{-6})	EC_{50}^{c} (μM)	95% Confidence limit (10^{-6})
Fluodipine								
Guinea pig	90 ± 3.1	63 ± 3.0	0.011	0.009–0.012	0.020	0.016–0.026	0.096	0.07–0.12
Rat	76 ± 3.2[d]	36 ± 2.5	0.240	0.17–0.32	0.830	0.74–0.95	–	–
Rabbit	97 ± 2.8[e]	52 ± 2.8	0.170	0.11–0.24	0.780	0.67–0.93	0.430	0.35–0.52
Nifedipine								
Guinea pig	85 ± 4.2	97 ± 2.0	0.020	0.01–0.03	0.039	0.03–0.05	0.270	0.20–0.30
Rat	83 ± 5.8[d]	86 ± 1.2[e]	0.216	0.17–0.28	0.440	0.34–0.55	0.116	0.09–0.16
Rabbit	95 ± 4.5[f]	91 ± 2.3	0.120	0.09–0.16	0.330	0.25–0.42	0.450	0.38–0.53

[a] Pretreatment heart rate ranged from 165 to 190 beats min^{-1}.
[b] The left atria were driven at 1 Hz.
[c] Calculated from log concentration–response curves (Probit analysis according to Litchfield and Wilcoxon with $n = 5–7$).
[d] At 5×10^{-6} M.
[e] At 10^{-5} M. At this concentration, fluodipine (five or six out of eight experiments) produced complete standstill.
[f] At 10^{-6} M. At this concentration nifedipine (four or five out of seven experiments) produced complete standstill.
When the maximum effect was $< 50\%$, the EC_{50} inotropic value was not calculated.

Table 2
Effect on vascular contraction

Species	Effect on K^+-depolarized aortic strips ($n = 5–6$) at 10^{-6} M	IC_{50} on vascular contraction	
	% Inhibition of contraction (mean ± S.E.M.)	IC_{50}^a (μM)	95% Confidence limit (10^{-6})
Fluodipine			
Guinea pig	54 ± 3.8[b]	1.400	1.1–1.7
Rat	85 ± 2.1[c]	0.052	0.04–0.06
Rabbit	51 ± 1.6[d]	1.460	1.1–1.9
Nifedipine			
Guinea pig	82 ± 1.3	0.0090	0.003–0.02
Rat	94 ± 5.5[e]	0.0053	0.004–0.007
Rabbit	82 ± 5.4	0.024	0.019–0.030

[a]Calculated from log concentration–response curves (Probit analysis according to Litchfield and Wilcoxon with $n = 5–7$).
[b]At 10^{-4} M.
[c]At 5×10^{-6} M.
[d]At 5×10^{-5} M.
[e]At 10^{-7} M.

2.5. Drugs

The following drugs were used: ketalar [(±) ketamine hydrochloride] (Parke Davis); pentobarbital [5-ethyl-5-[1-methylbutyl]-2,4,6-trioxohexahydro-pirimidine sodium salt] (Sigma); nifedipine [1,4-dihydro-2,6-dimethyl-4-(*o*-nitrophenyl)pyridine-3,5-dicarboxylic acid dimethyl ester] (Sigma); fura-2 acetoxymethyl ester (Sigma); fluodipine [1,4-dihydro-2,6-dimethyl-4-(fluorenon-4-yl)pyridine-3,5-dicarboxylic acid diallyl ester] was synthesized in the laboratory of the Department of Pharmaceutical Sciences, University of Bologna.

3. Results

3.1. In vitro experiments

3.1.1. Effect on chronotropism in spontaneously beating right atria

The chronotropic and inotropic activities of nifedipine and fluodipine are reported in Table 1. Fluodipine was the most effective bradycardizing agent in guinea pig atria, its EC_{30} on chronotropism being about half that of nifedipine. Furthermore fluodipine showed the highest species selectivity, being about from 15 to 22 times more potent on guinea pig than on rat and on rabbit right atria.

3.1.2. Effect on inotropism in electrically driven left atria

Considering negative inotropic activity, fluodipine appeared to be the most active on guinea pig and rabbit driven left atria. It was about 2.5–3 times more potent than nifedipine on guinea pig preparations. It showed the highest species selectivity, being about 4.5 times more potent on guinea pig than on rabbit left atria. Fluodipine was less effective than nifedipine on rat left atria.

3.1.3. Effect on vascular contraction in aortic strips

The vasorelaxing activity of the compounds, tested on K^+-depolarized aortic strips, is reported in Table 2. Fluodipine was much less effective than nifedipine to inhibit contraction in guinea pig and in rabbit preparations (about 155 and 60 times less effective, respectively).

Table 3
Comparison of drugs effects on chronotropism, inotropism and vascular contraction in various species

Species	EC_{50} (nM) on chronotropism (spontaneously beating right atria)	EC_{50} (nM) on inotropism (electrically driven left atria)	IC_{50} (nM) on vascular contraction (K^+-stimulated aorta)	Ratio (chronotropic/vascular contraction)	Ratio (inotropic/vascular contraction)
Fluodipine					
Guinea pig	20	96	1400	0.014	0.07
Rat	830	–	52	15.96	ND
Rabbit	780	430	1460	0.53	0.29
Nifedipine					
Guinea pig	39	270	9.0	4.33	30
Rat	440	116	5.3	83.02	21.89
Rabbit	330	450	24	13.75	18.75

ND = not determined since EC_{50} inotropic value was not calculated according to the footnote to Table 1.

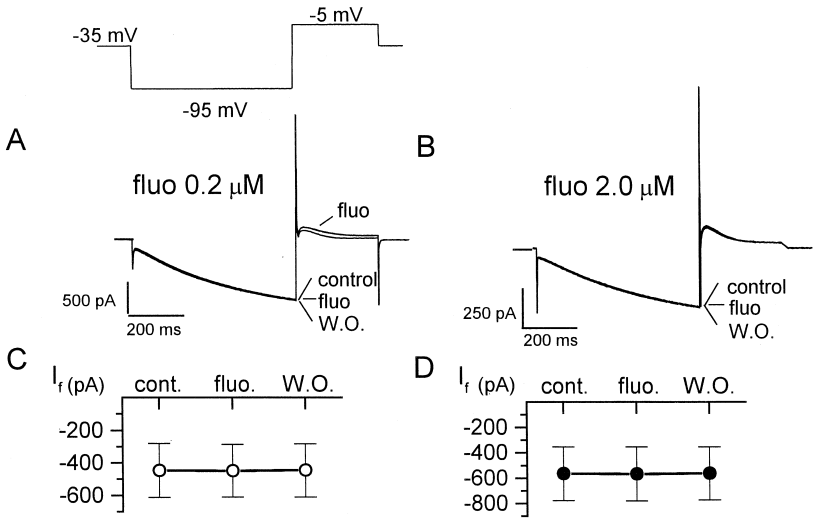

Fig. 2. Effect of fluodipine on the pacemaker current I_f recorded in isolated rabbit sino-atrial myocytes. Contamination by K^+ and Ca^{2+} currents was removed with 1 mM Ba^{2+} and 2 mM Mn^{2+}, respectively. The protocol step used to activate I_f is shown in the upper left panel. (A) I_f recording in control, during superfusion of fluodipine 0.2 mM (fluo), and after washout (W.O.). The small divergence of current traces during the pulse at −5 mV can be attributed to a small, accidental change in background conductance; the amplitude of tail current, reflecting I_f deactivation, was indeed unmodified by the drug. Such a change was not consistently observed in different cells. (B) I_f recording in control, during superfusion of fluodipine 2.0 mM (fluo), and after washout (W.O.). (C) Average (±S.E.M.) results obtained in three cells superfused with fluodipine 0.2 mM. (D) Average (±S.E.M.) results obtained in four cells superfused with fluodipine 2.0 mM.

To compare the potency and the tissue selectivity of the compounds on the basis of equivalent pharmacological parameters, we show in Table 3 the EC_{50} and IC_{50} values estimated for cardiac and vascular preparations from the various species examined. The ratio of EC_{50} and IC_{50} shows a clear-cut affinity and selectivity of fluodipine for guinea pig and rabbit cardiac tissue. With rat preparations, fluodipine was more potent at the vascular than at the cardiac level.

3.1.4. Effect on the pacemaker current I_f in isolated sino-atrial node myocytes

The ionic mechanism of the bradycardic action of the compound was studied in rabbit isolated sino-atrial node myocytes, by means of whole-cell patch-clamp recording. We tested whether the bradycardic action was at least partially due to inhibition of the hyperpolarization-activated inward current I_f, which is involved in the generation of pacemaker activity and in its modulation by acetylcholine and catecholamines (Di Francesco et al., 1986, 1989). The results obtained seem to exclude this possibility: as shown in Fig. 2, the mean current amplitude, measured at the end of a 600-ms hyperpolarizing current pulse, was not significantly changed in myocytes perfused with a solution containing fluodipine 0.2 μM (448.5 ± 162.6 pA vs. a control of 446.7 ± 166.7 pA, $n = 3$ N.S.) or 2 μM (567.0 ± 213.1 pA vs. a control of 564.9 ± 212.2 pA, $n = 4$ N.S.). The current was unaffected by superfusion with the vehicle, dimethylformammide, alone.

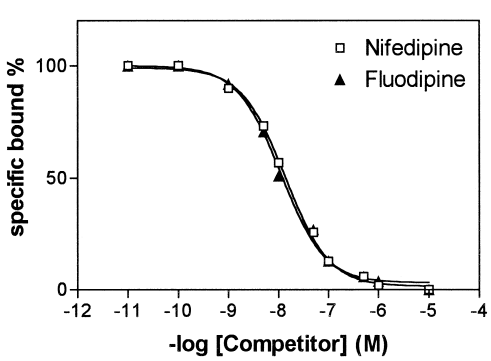

Fig. 3. Representative competition binding isotherms for [^{3}H]isradipine binding by fluodipine (▲) and nifedipine (□) in guinea pig heart membranes. Each data point represents the mean of two determinations. The curves are from a representative experiment repeated at least three times.

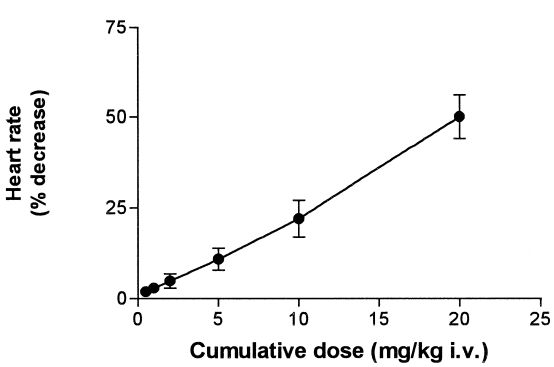

Fig. 4. Effect of incremental doses of fluodipine (●) on heart rate in anesthetized rabbits. Values represent the means ± S.E.M. ($n = 5$) of changes calculated as percentages of the control value recorded immediately before drug administration.

Table 4
Effect of fluodipine on mean arterial pressure in anesthetized rabbits

Dose (mg kg^{-1}, i.v.)	Mean arterial pressure $n = 5$ (mmHg, mean ± S.E.M.)
0	133 ± 10
0.5	128 ± 7
1.0	130 ± 5
2.5	118 ± 9
5.0	116 ± 8
10	110 ± 10
20	106 ± 11

3.1.5. Effect on Ca^{2+} influx in A_7r_5 aortic smooth muscle cells

Nifedipine and fluodipine produced a dose-dependent inhibition of cytosolic Ca^{2+} elevation in cells exposed to KCl, and fluodipine was the least effective. The IC_{50} were 11.05 + 0.28 nM ($n = 3$) for nifedipine and 792 + 32 nM ($n = 3$) for fluodipine.

3.1.6. Binding to cardiac calcium channels

Displacement binding studies on guinea pig ventricular homogenates labeled with [^{3}H]Isradipine showed that the fluodipine affinity to L-type cardiac calcium channels (K_i = 2.57 ± 0.05 nM; $n = 3$) was similar to that of nifedipine (K_i = 1.99 ± 0.03 nM; $n = 3$) (Fig. 3).

3.2. In vivo experiments

3.2.1. Effect on chronotropism and on arterial pressure in the anesthetized rabbit

Fluodipine decreased the spontaneous heart rate of anesthetized rabbits when given as cumulative doses (Fig. 4). These bradycardic responses were dose-related: the calculated ID_{25} was 8.5 mg kg^{-1}, i.v. The onset of bradycardia was observed within 1–2 min and it plateaued by 5 min. The duration of cardiac slowing was not followed longer than from the 10-min interval. A slight decrease in mean arterial pressure was observed only with the higher doses (Table 4) and was not statistically significant ($P > 0.05$; Duncan's test after ANOVA). In separate experiments, nifedipine (0.05–20 mg kg^{-1}, i.v.) was taken as a reference calcium antagonist; it induced a significant reduction of mean arterial pressure (ID_{25} = 0.18 mg kg^{-1}, i.v.) whereas heart rate was not significantly reduced (data not shown). In preliminary experiments carried out in conscious rabbits, fluodipine (10 mg kg^{-1}, i.v.) significantly decreased the spontaneous heart rate in comparison to that of vehicle-treated animals (199.8 ± 5.2 vs. 259 ± 6.8 beats min^{-1}, $P < 0.05$; $n = 4$).

4. Discussion

1,4-Dihydropyridines owe their therapeutic usefulness to the interaction at the L-type voltage-dependent Ca^{2+} channels controlling several functions of the cardiovascular system. These agents, particularly the second generation 1,4-dihydropyridines structurally related to nifedipine (Triggle, 1996), have a strong vascular vs. cardiac selectivity. A number of factors contribute to tissue selectivity, including types and subtypes of Ca^{2+} channels, pharmacokinetic properties and the use-dependence of antagonist actions on channels (Godfraind, 1994; Triggle, 1996).

The results now obtained with atria and aorta in vitro preparations demonstrate that fluodipine is characterized by a tissue selectivity very different from that of the other 1,4-dihydropyridines, i.e., similar affinity for cardiac Ca^{2+} channels and much lower efficacy on vascular Ca^{2+} channels. As expected (Godfraind, 1994), we found significant species differences in tissue selectivity. The most selective cardiodepressant activity was observed on guinea pig preparations, where fluodipine was about 2–3 times more potent than nifedipine at the cardiac level and about 150 times less effective on vascular tissue. Similarly to nifedipine, fluodipine showed the lowest cardiac selectivity on rat preparations; this supports the view that 1,4-dihydropyridines may have a relatively low affinity for rat cardiac Ca^{2+} channels (Kazda et al., 1980; Finet et al., 1985; Boyd et al., 1988; Wibo et al., 1988; Spedding et al., 1990; Godfraind, 1994).

While negative inotropic effects of fluodipine may confidently be attributed to calcium channel blockade, other mechanisms might account for the pronounced bradycardic effect. However, two lines of evidence suggest that both drug effects may be based on the same action, i.e., Ca^{2+} channel blockade: (i) for each compound, bradycardia and Ca^{2+} blocking activity at cardiac sites (negative inotropism) were observed within the same concentration range; (ii) patch-clamp studies on isolated sino-atrial node myocytes ruled out an effect of fluodipine on I_f, the other current whose inhibition might lead to the observed effects on rate.

The observations with isolated atria and aorta preparations are consistent with the results obtained in (i) binding experiments with guinea pig ventricular homogenates, showing that fluodipine and nifedipine have a similar affinity to L-type cardiac Ca^{2+} channels; (ii) fura-2 experiments with rat A_7r_5 vascular smooth muscle cells, in which fluodipine was about 80 times less effective than nifedipine to inhibit cytosolic Ca^{2+} elevation in response to KCl exposure.

Fluodipine and nifedipine displayed a similar potency in binding experiments whereas fluodipine was about 2–3 times more potent on in vitro guinea pig heart preparations. This difference could be explained by taking into account that the interaction of 1,4-dihydropyridines with L-type Ca^{2+} channels is state-dependent, in that their affinity and/or access to drug binding sites is dependent on the state of the channel as influenced by membrane potential, phosphorylation state and other factors

(Hondeghem and Katzing, 1984); and that, on the contrary, receptor binding experiments are done with disrupted membrane fragments. Therefore these state-dependent interactions may influence the pharmacological profile of fluodipine evaluated in in vitro and in vivo models. In agreement with the results obtained with in vitro rabbit preparations, we found that fluodipine induces a significant reduction of the spontaneous heart rate in the anesthetized rabbit, without affecting blood pressure. Duncker et al. (1986) have reported that i.v. injection of the 1,4-dihydropyridine derivatives, nimodipine and nisoldipine, in conscious pigs provokes a fall in arterial blood pressure; it may also cause an increase of heart rate and cardiac output as consequence of reflex activation of the sympathetic nervous system via the baroceptors, but this effect is masked by pentobarbitone anaesthesia. As regards fluodipine, we ruled out the hypothesis that an increase of heart rate could be blunted by the anaesthetic agent, indeed, fluodipine did not induce a significant reduction of mean arterial pressure in the anesthetized rabbit, and did not elevate heart rate in conscious rabbit. This profile of activity indicates that this compound may be included among the bradycardic, non-vasculating agents, useful when bradycardia not associated with vasodilatation is required. As suggested by the greater efficacy of verapamil and diltiazem vs. 1,4-dihydropyridines on exercise-induced angina, a Ca^{2+} channel blocking agent with strongly preponderant cardiac vs. vascular effects may have a significant therapeutic advantage whenever the ischemia is not primarily a consequence of coronary spasm. Indeed, while a reduction in sinus rate and oxygen consumption, actually resulting from decreased adrenergic stimulation, may also be obtained by β-adrenoceptor blockade, the clinical use of β-adrenoceptor antagonists is limited, in relatively large subsets of patients, by their antagonism of β-adrenoceptor-mediated bronchodilatation and control of glucose blood levels. These limitations do not apply for fluodipine, making the compound potentially useful in patients with concomitant obstructive lung disease or under insulin therapy. A pure bradicardic action may also be obtained with blockers of the sinoatrial pacemaking current I_f, but the clinical use of currently available compounds is limited by their ocular toxicity (Frishman et al., 1996). Since fluodipine does not affect I_f (present observations), this side-effect should not be present.

In conclusion, the present results demonstrated that fluodipine has selective cardiodepressant activity, and suggest that this effect is due to a higher affinity for cardiac than for vascular L-type Ca^{2+} channels. According to the pharmacodynamic profile, as defined by present in in vitro and in vivo observations, fluodipine might represent a useful tool in the treatment of ischemic heart disease, particularly in patients with preserved contractility and with contraindications for β-adrenoceptor blockade. Thus, further evaluation of fluodipine effects in experimental models of ischemic heart disease in vivo seems worthwhile.

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