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A. L , L. H -M , J. H R. F . Sympathetic Modulation of the Effect of Nifedipine on Myocardial Contraction and Ca Current in the Rat. *Journal of Molecular and Cellular Cardiology* (1997) **29**, 579–591. The regulation of cardiac -type Ca^{2+} current (I_{Ca}) and contraction by dihydropyridine antagonists and β-adrenergic receptor agonists has been the subject of numerous studies over the last decade. However, little is known on the crosstalk between these two regulatory pathways. For instance, a fundamental question that remains unanswered is: does activation of the β-adrenergic receptors modify the sensitivity of the myocardium to dihydropyridine agonists? To answer this question, we examined in the present study how activation of the β-adrenergic receptors modifies the effects of nifedipine on the mechanical and energetic parameters of the isolated perfused rat heart. Activation of the β-adrenergic receptors was achieved by perfusing the hearts with isoprenaline, a non-selective β-adrenergic receptor agonist, and could be reduced by atenolol, a β_1-adrenergic receptor antagonist. To examine possible alterations during hypertension in the sensitivity of the hearts to the drugs tested, the study was performed in both normotensive Wistar–Kyoto (WKY) and spontaneously hypertensive animals (SHR). While 0.1 μ nifedipine reduced left ventricular pressure (LVP) by 36% and 34% in WKY and SHR rats, respectively, under basal conditions, its effects became negligible in both groups of rats after stimulation of the hearts with 0.1 μ isoprenaline. Addition of 1 μ atenolol in the presence of isoprenaline restored the inhibitory effect of nifedipine to control values in both WKY and SHR rats. Additional experiments were performed in isolated ventricular myocytes from WKY rats using the whole-cell patch-clamp technique. The inhibitory effects of 0.1 to 1 μ nifedipine were significantly larger on basal I_{Ca} than after the current had been previously elevated by 0.1 μ isoprenaline. Addition of 1 μ atenolol in the presence of isoprenaline partially restored the inhibitory effect of nifedipine on I_{Ca}. Our results demonstrate a reduced sensitivity of the heart muscle to nifedipine during activation of β_1-adrenergic receptors. This effect is partly explained by a reduced inhibitory effect of nifedipine on I_{Ca} during activation of cAMP-dependent phosphorylation.

K W : Contraction; Perfused heart; Ca^{2+} Current; Cardiomyocyte; Nifedipine; Dihydropyridine ant-agonists; Atenolol; β-Adrenergic agonists; β-Adrenergic antagonists.

Introduction

1,4-dihydropyridine (DHP) derivatives have been extensively studied for their potent inhibitory effects on the -type Ca^{2+} channel current (I_{Ca}), particularly in vascular and cardiac muscle cells. Biochemical, electrophysiological and functional studies have provided ample information on the mechanisms of

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action of these drugs and their physiological effects (Porzig, 1990). In isolated preparations from vascular or cardiac muscles, DHP antagonists produce a direct inhibition of contraction which correlates well with the inhibition of I_{Ca}. However, *in vivo*, when used at therapeuutic doses, DHP antagonists act preferentially to relax the vascular smooth muscle cells and reduce blood pressure (Godfraind, 1994). This tissue selectivity of DHP antagonists may be partly explained by the fact that vascular smooth muscle cells rest at a less negative membrane potential and depolarize for a longer time than cardiac myocytes. This makes them more sensitive to DHP antagonists, because depolarization of the membrane potential increases the affinity of DHP antagonists for the -type Ca^{2+} channels strongly (Porzig, 1990) and enhances their inhibitory effect on I_{Ca} (Godfraind and Govoni, 1989; Godfraind, 1994). In addition, slight differences exist in the molecular structure of α_1 subunits of cardiac and vascular smooth muscle -type Ca^{2+} channels which confer to the latter a higher sensitivity to DHP antagonists (Welling *et al.*, 1993). Thus, *in vivo*, the direct inhibitory effect of DHP antagonists on cardiac I_{Ca} is smaller than on vascular I_{Ca}.

Another component that may contribute to the tissue selectivity of DHP antagonists is the different sensitivity of cardiac and vascular smooth muscle Ca^{2+} channels to β-adrenergic agonists. While in cardiac muscle, activation of β-adrenergic receptors leads to a strong stimulation of -type Ca^{2+} channel activity, and hence contraction, via activation of cAMP-dependent phosphorylation (Hartzell and Fischmeister, 1992; McDonald *et al.*, 1994), this pathway plays a minor (McDonald *et al.*, 1994) or controversial role (Xiong and Sperelakis, 1995; Shi and Cox, 1995) in the regulation of vascular smooth muscle Ca^{2+} channels. Because the reduction in blood pressure induced by DHP antagonists is generally accompanied by the activation of a baroflex which results in an increased release of noradrenaline at the cardiac junction (Piepho, 1995), cardiac muscle -type Ca^{2+} channels are likely to be activated by this pathway. Thus, cardiac -type Ca^{2+} channels, unlike smooth muscle channels, are dually regulated by two opposite mechanisms: a direct inhibition by DHP antagonists and an indirect stimulation via activation of β-adrenergic receptors.

Surprisingly, although each type of regulation has been studied in detail on both cardiac I_{Ca} and contraction, little is known on the crosstalk between the two regulatory pathways. For instance, a fundamental question that remains unanswered is: does activation of the β-adrenergic signaling pathway modify the sensitivity of the myocardium to DHP antagonists? To get some insights into this question, we examined in the present study how activation of the β-adrenergic receptors modifies the effects of nifedipine on the mechanical and energetic parameters of the isolated perfused rat heart. Activation of the β-adrenergic receptors was achieved by perfusing the hearts with isoprenaline, a non-selective β-adrenergic receptor agonist, and could be reduced by atenolol, a β_1-adrenergic receptor antagonist. Because DHP antagonists are mainly used to treat hypertension (Godfraind, 1994) and hypertension modifies the control of myocardial function by β-adrenergic agonists (Moravec *et al.*, 1995), the study was performed in both normotensive Wistar–Kyoto (WKY) and spontaneously hypertensive animals (SHR). Additional experiments were performed in isolated ventricular myocytes from WKY rats using the whole-cell patch-clamp technique to examine the regulation of I_{Ca} by nifedipine in the absence or presence of isoprenaline and/or atenolol. All our results converge towards a reduced sensitivity of the heart muscle to nifedipine during activation of β-adrenergic receptors. This may be partly explained by a reduced inhibitory effect of nifedipine on -type Ca^{2+} channels during activation of cAMP-dependent phosphorylation. Some preliminary results have appeared in abstract form (Legssyer *et al.*, 1995).

Materials and Methods

The investigation conforms with the European Community guiding principles in the care and use of animals (86/609/CEE, *CE Off J* n°L358, 18 December 1986) and the French decree n°87/748 of October 19, 1987 (*J Off République Française*, 20 October 1987, pp. 12245–12248). Authorizations to perform animal experiments according to this decree were obtained from the French Minstère de l'Agriculture et de la Forêt (n°04225 to JH and 04226 to RF, April 12, 1991).

Animal experimentation

Animal experimentation was performed in accordance with the American Heart Association's position statement on research animal usage. Male SHR and normotensive WKY rats were age matched (12–13 weeks old). The characteristics of the animals are summarized in Table 1. Body weight was lower in SHR. The left and right ventricles and the

Table 1 Morphological characteristics of the animals

	Body weight	Heart weight	Left ventricle weight	Right ventricle weight	Kidney weight
WKY, $n=6$					
Absolute values (g)	367 ± 13	1.35 ± 0.08	0.94 ± 0.02	0.29 ± 0.01	2.57 ± 0.08
Relative to body weight (mg/g)		3.56 ± 0.14	2.51 ± 0.12	0.78 ± 0.03	7.29 ± 0.17
SHR, $n=7$					
Absolute values (g)	320 ± 5*	1.40 ± 0.04	1.09 ± 0.04*	0.27 ± 0.01	2.25 ± 0.05
Relative to body weight (mg/g)		4.30 ± 0.08*	3.21 ± 0.08*	0.88 ± 0.05	7.03 ± 0.19

The data indicate the mean $\pm$ . . . of the number of hearts n given in the first column except for kidney weight (WKY: $n=4$; SHR: $n=3$). Statistically significant differences between SHR and WKY are indicated as * at the $P<0.01$ level (t-test).

kidneys were weighed at the end of the experiments. Heart weight was similar in SHR and WKY rats but the increased heart weight to body weight ratio in SHR rats showed the development of hypertrophy in both ventricles from these animals.

Isolated heart perfusion

After anesthesia of the rats with 2 g/kg ethylcarbamate, the hearts were removed and perfused by the Langendorff technique at a constant flow of 12 ml/min. The left ventricle (LV) was pierced to avoid fluid accumulation. A latex balloon was inserted in the LV and allowed continuous recording of mechanical parameters via a Statham gauge (Palo Alto, CA, USA). After 10 min of stabilization, the balloon was progressively inflated to isovolumic condition (LV volume *c.* 80 μl). The end diastolic pressure was 5–8 mmHg. A second pressure gauge was used to record the coronary pressure just above the aorta. The pulmonary artery was cannulated and its effluent was analyzed for oxygen content.

The perfusion solution contained (in m): NaCl 113; KCl 4.7; $NaHCO_3$ 25; $MgSO_4$ 1.2; $CaCl_2$ 1.5; KH_2PO_4 1.2; mannitol 1.2; Na-pyruvate 5; pH was adjusted to 7.4 with NaOH and the solution was equilibrated at 37°C by 95% O_2–5% CO_2. Nifedipine was infused via a teflon catheter just above the aorta. Preliminary experiments showed that 10 n nifedipine had no effect on contractile parameters of the isolated perfused hearts. Thus, we routinely used nifedipine at a concentration of 0.1 μ . Atenolol, a β_1-adrenergic receptor antagonist, and isoprenaline, a non-selective β-adrenergic receptor agonist, were added to the perfusate at concentrations of 0.1 or 1 μ and 0.02 or 0.1 μ , respectively. Fresh solutions were prepared before each experiment and the whole circuit down to the aorta was protected from light by aluminium foil.

After 10 min of stabilization in isovolumic conditions of work, the experiments started by transient applications of the drugs. Nifedipine was applied alone, or in the presence of isoprenaline and/or atenolol. The effects of nifedipine in these different experimental conditions were analysed in seven WKY and seven SHR rats.

Data analysis

Oxygen consumption and mechanical parameters were analyzed using a computer program developed in the laboratory which allowed us to average four to ten consecutive contractions. Systolic activity was evaluated by the left ventricular pressure development (LVP in mmHg), the heart rate (HR in beats/min) and an index of work: the rate pressure product (RPP = rate $\times$ LVP, in 10^4 mmHg beats/min). Relaxation parameters were given by the time for half relaxation ($t_{\frac{1}{2}}$ in ms) and the maximal rate of relaxation normalized to the peak of contraction [$-(dLVP/dt)_{max}$/LVP in s^{-1}]. Other measured contractile parameters included the time to peak of contraction (TTP in ms), and the maximal rate of contraction activation normalized to the peak of contraction [$+(dLVP/dt)_{max}$/LVP in s^{-1}]. Oxygen consumption (Q_{O_2} in μmol O_2/min/g wet weight) was measured as the difference between oxygen partial pressure (P_{O_2}) of the perfusate at the level of the aorta ($P_{O_{2A}}$) and the pulmonary artery ($P_{O_{2,v}}$). Both P_{O_2}s were continuously measured in thermostated chambers at constant flow (0.8 ml/min) using small Clark electrodes and Strathkelvin oxymeters (Strathkelvin Instruments, Glasgow, UK). Typically, the time for 95% response of the electrode was 20 s. The mean diastolic coronary pressure (CP in mmHg) was obtained by averaging the coronary pressure during several cardiac cycles.

Electrophysiology

Cardiac myocytes were obtained by retrograde perfusion of the heart as previously described (Pucéat *et al.*, 1990). The ionic composition of the solutions were as follows. Ca-free solution (in m): NaCl 117; KCl 5.7; $NaHCO_3$ 4.4; KH_2PO_4 1.5; $MgCl_2$ 1.7; HEPES 21.1; creatine 10; -glucose 11.7; taurine 20; pH adjusted to 7.1 with NaOH at room temperature. For enzymatic dissociation 1 mg/ml collagenase A (Boehringer-Mannheim, Germany) and 300 μ EGTA were added to the Ca-free solution, and the free Ca concentration was then adjusted to 20 μ . After dissociation, the cells were kept at 37°C and pH 7.4 in the Ca-free solution to which was added 1 m Ca and 0.5 mg/ml fatty acid-free bovine serum albumin. Cells were used between 1 and 8 h after dissociation for electrophysiological experiments. The -type Ca current (I_{Ca}) was measured with the patch-clamp technique in the whole-cell configuration, using an EPC-7 patch-clamp amplifier (List instruments, Darmstadt, Germany) and custom built software for acquisition of peak L_{Ca} as well as the current at the end of the pulse and the holding current (Fischmeister and Hartzell, 1986). The holding potential was -80 mV, and I_{Ca} was elicited every 8 s by 400-ms voltage pulses from -80 to 0 mV, delivered by a programmable digital stimulator (Challenger DB, Kinetic Software Atlanta, Atlanta, GA USA). The control external solution contained (in m): 107 NaCl; 20 CsCl; 4 $NaHCO_3$; 0.8 NaH_2PO_4; 1.8 $MgCl_2$; 10 HEPES; 5 -glucose; 5 Na-pyruvate; 0.03 tetrodotoxin (Latoxan, Rosans, France); pH was adjusted to 7.4 with NaOH at room temperature. Patch electrodes (0.6–1.2 MΩ) were made from soft or hard glass and filled with control internal solution containing (in m): 119.8 CsCl; 5 EGTA; 4 $MgCl_2$; 5 creatine phosphate disodium salt; 3.1 Na_2ATP; 0.42 Na_2-GTP; 0.062 $CaCl_2$ (pCa = 8.5); 10 HEPES; pH adjusted to 7.3 with CsOH at room temperature. Stock solutions of nifedipine, isoprenaline and atenolol were freshly prepared every day and diluted to the desired concentration immediately before application to the cell. To avoid light degradation of the stock solutions, these were kept in plastic tubes wrapped in aluminium foil. Nifedipine was dissolved in pure (96%) ethanol, but the final ethanol concentration (less than 0.1%) did not in itself affect I_{Ca}. All drugs were from Sigma (St Louis, MO, USA) unless specified.

The cells were perfused by placing the cell at the opening of a capillary with an inner diameter of 300 μm. Solutions were changed by moving the cell to the opening of an adjacent capillary containing the desired solution. The flow rate in the capillaries (around 10 μl/min) was regulated by gravity. All patch-clamp experiments were performed at room temperature (22–26°C).

Statistical analysis

Results are expressed as mean $\pm$ . . . In each experimental condition, the effects of nifedipine on contractile and energetic parameters or on I_{Ca} are expressed as percentage change with respect to the values of these parameters in the absence of nifedipine. Statistical differences for the effect of nifedipine in each experimental condition and group of animals were evaluated using the Student's paired *t*-test. Statistical differences for the effects of atenolol and isoprenaline between SHR and WKY rat hearts were analyzed using variance analysis and Bonferroni *t*-test.

Results

Effect of nifedipine and atenolol under basal conditions in WKY rats

After 30 min of equilibration in isovolumic conditions of work, the perfused WKY rat hearts stabilized their contractile and energetic parameters to the control values given in Table 2. After this period, transient infusions of nifedipine, atenolol, or a combination of both were tested. Figure 1 shows a typical experiment performed on a WKY rat. In this experiment, the effects of 0.1 μ nifedipine were first examined alone. Nifedipine produced a profound reduction of LVP. The maximum effect was reached after 2 min of infusion, and was fully reversible upon wash-out of the drug [Fig. 1(a)]. Contractile activation was not affected by nifedipine but relaxation was slightly slower as shown on the individual contraction traces of Figure 1(b). Table 2 summarizes the effects of nifedipine on contractile and energetic parameters in WKY rats. Nifedipine decreased systolic pressure markedly and, as a result, RPP and oxygen consumption. Both the changes in time of half relaxation and rate of relaxation confirmed a significant slowing down of the relaxation. However, heart rate, coronary pressure and contractile activation parameters were not affected by the drug.

The effects of nifedipine were then examined in the presence of 0.1 μ atenolol (Fig. 1). Atenolol *per se*, at a concentration of 0.1 or 1 μ (not

Table 2 Effects of nifedipine (NIF), atenolol (ATE), and their combination on the mechanical and energetic parameters of the WKY perfused rat heart under basal conditions and in the presence of 0.1 μ isoprenaline (ISO)

	Basal conditions				Isoprenaline (0.1 μ)			
	control ($n=14$)	NIF 0.1 μ ($n=7$)	ATE 0.1 μ ($n=7$)	ATE 0.1 μ + NIF 0.1 μ ($n=4$)	ISO alone ($n=4$)	+ NIF 0.1 μ ($n=4$)	+ ATE 1 μ ($n=4$)	+ ATE 1 μ + NIF 0.1 μ ($n=4$)
	Absolute values	% change	Absolute values	% change	Absolute values	% change	Absolute values	% change
LVP	109 ± 6	−36 ± 2*	105 ± 12	−29 ± 4*	114 ± 5	−5 ± 2¶	76 ± 12§	−36 ± 9*
$+[dLVP/dt]_{max}/LVP$	24 ± 1	+0 ± 1	25 ± 2	+0 ± 2	43 ± 1‡	−1 ± 1	31 ± 1‡§	−5 ± 3
TTP	72 ± 2	+2 ± 1	66 ± 3	+3 ± 2	34 ± 2‡	+2 ± 2	52 ± ‡2§	+8 ± 3*
$-[dLVP/dt]_{max}/LVP$	16.9 ± 0.4	−10 ± 2*	17.6 ± 0.4	−6 ± 2	32 ± 1‡	+0 ± 1§	23 ± 1‡§	−19 ± 3*¶
$T_{1/2R}$	62 ± 1	+8 ± 2*	58 ± 1	+2 ± 3	35 ± 2‡	+2 ± 2	41 ± 2‡	+12 ± 3
HR	244 ± 9	−3 ± 3	236 ± 19	+1 ± 1	350 ± 8‡	+1 ± 4	285 ± 10§	−6 ± 3
RPP	2.6 ± 0.1	−38 ± 2*	2.4 ± 0.2	−28 ± 4*	3.8 ± 0.1‡	−3 ± 2¶	2.1 ± 0.3§	−40 ± 7*
CP	47 ± 4	−3 ± 6	61 ± 7†	−6 ± 6	62 ± 4‡	+1 ± 1	67 ± 6	+5 ± 2
Q_{O_2}	6.0 ± 0.3	−20 ± 1*	7.0 ± 0.8	−17 ± 2*	9.2 ± 0.5‡	−3 ± 1*¶	7.0 ± 0.7§	−24 ± 3*

NIF: nifedipine: ATE: atenolol; ISO: isoprenaline; LVP: maximum systolic left ventricular pressure (in mmHg); $+[dLVP/dt]_{max}/LVP$: maximal rate of rise of contraction normalized to LVP (in s^{-1}); TTP: time to peak of contraction (in ms); $-[dLVP/dt]_{max}/LVP$: maximal rate of relaxation normalized to LVP (in s^{-1}); $T_{1/2R}$; time of half relaxation (in ms); HR: heart rate (in beats/min); RPP: rate pressure product (=LVP.HR in 10^{-4} mmHg/beats/min); CP: coronary pressure (in mmHg); Q_{O_2}; oxygen consumption (in μmol O_2/min/g w.w.). The data indicate the mean ± . . . of the number of hearts n given in the parentheses. Statistically significant variations induced by NIF are indicated as: * $P<0.05$. The only significant effect of ATE as compared to control is indicated as †($P<0.05$). When the absolute values obtained in ISO alone or in ISO + ATE differed significantly from the control absolute values, they are labeled as ‡($P<0.01$). The statistically significant differences between the values in ISO alone and in ISO + ATE are indicated as §($P<0.05$). When the effects of NIF in ISO alone or in ISO + ATE were significantly different from the effects of NIF under basal conditions, they are indicated as ¶($P<0.05$).

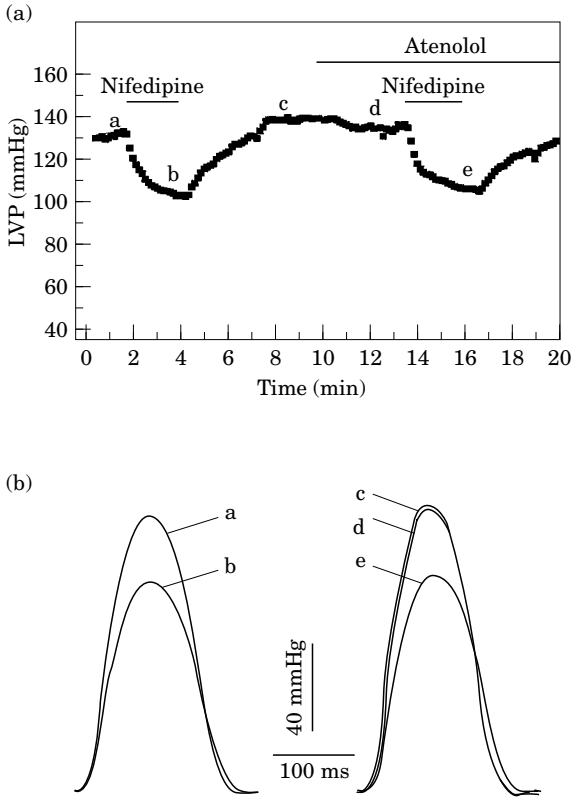

Figure 1 (a) Time course of the effect of nifedipine in the absence or presence of atenolol on the left ventricular pressure (LVP) of an isolated perfused heart from a WKY rat. After 30 min of equilibration in isovolumic conditions of work, nifedipine (0.1 μ) was applied during the period indicated and produced a clear negative inotropic effect. After wash-out of nifedipine, LVP returned to control amplitude. Atenolol (0.1 μ) was subsequently applied during the period indicated and produced no change in LVP. A second application of 0.1 μ nifedipine induced a similar effect on LVP as in the absence of atenolol. (b) Individual contraction traces were obtained at the times indicated by the corresponding letters on the graph in (a).

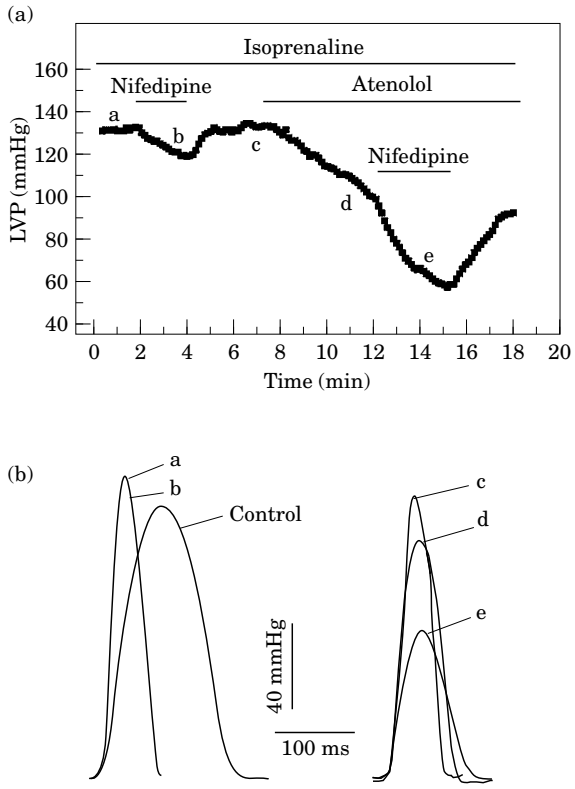

Figure 2 (a) Time course of the effect of nifedipine in the presence of isoprenaline and in the absence or presence of atenolol on the left ventricular pressure (LVP) of an isolated perfused heart from a WKY rat. After 30 min of equilibration in isovolumic conditions of work, the heart was perfused with isoprenaline (0.1 μ) throughout the rest of the experiment. In the presence of isoprenaline, nifedipine (0.1 μ) was applied during the period indicated and produced a small negative inotropic effect. After wash-out of nifedipine, LVP returned to its amplitude in isoprenaline alone. Atenolol (1 μ) was subsequently applied throughout the rest of the experiment and produced a profound inhibition of LVP. A second application of 0.1 μ nifedipine on top of isoprenaline + atenolol induced a large and reversible effect on LVP. (b) Individual contraction traces were obtained in the absence of isoprenaline (control) and at the times indicated by the corresponding letters on the graph in (a).

shown), had no effect on LVP [Fig. 1(a)], time course of contraction and relaxation [*cf.* traces c and d in Fig. 1(b)], or other contractile or energetic parameters (Table 2). Moreover, the presence of atenolol did not modify the effects of nifedipine on most of these parameters (Table 2). However, the presence of atenolol somewhat attenuated, although not significantly, the effects of nifedipine on the parameters of relaxation (Table 2).

Effect of nifedipine and atenolol under β-adrenergic stimulation in WKY rats

Activation of β-adrenergic receptors with 0.1 μ isoprenaline in WKY rat hearts induced a transient increase in LVP to $177 \pm 24\%$ of its control value (mean $\pm$ . . ., $n=4$). This peak was observed ≈ 12 s after application of isoprenaline and was followed by a progressive return of LVP to control values. The steady state effects of 0.1 μ isoprenaline in WKY rat hearts are summarized in Table 2. As seen, isoprenaline induced a sustained rise in heart rate, a decrease in the time to peak of contraction, and an acceleration in the rates of contraction and relaxation [compare trace a with control trace in Fig. 2(b)].

As shown in Figure 2, the negative inotropic effect of 0.1 μ nifedipine was markedly reduced

Table 3 Effects of nifedipine on the mechanical and energetic parameters of the SHR perfused rat heart under basal conditions and in the presence of 0.1 μ isoprenaline

	Basal conditions		Isoprenaline 0.1 μ		
	Control ($n=19$)	Nifedipine 0.1 μ ($n=4$)	Isoprenaline ($n=6$)	+nifedipine 0.1 μ ($n=6$)	+atenolol 1 μ +nifedipine 0.1 μ ($n=4$)
	Absolute values	% change	Absolute values	% change	% change
LVP	139 ± 4†	-34 ± 5*	115 ± 8	-6 ± 4‡	-40 ± 3*
$+[dLVP/dt]_{max}/LVP$	22 ± 1	$+2\pm2$	44 ± 1*	0 ± 1	-3 ± 6
TTP	77 ± 3	$+2\pm2$	33 ± 1*	-1 ± 1	$+7\pm9$
$-[dLVP/dt]_{max}/LVP$	15.3 ± 0.4†	-9 ± 3*	29 ± 1*	-2 ± 3	-22 ± 7*
$T_{1/2R}$	71 ± 2†	$+6\pm2$*	35 ± 1*	$+2\pm1$	$+12\pm4$*
HR	205 ± 6†	$+2\pm2$	345 ± 9*	$+2\pm2$	-6 ± 8
RPP	2.8 ± 0.1	-33 ± 6*	4.0 ± 0.2*	-6 ± 4‡	-43 ± 7*
CP	62 ± 3†	-5 ± 2*	61 ± 2	-1 ± 1	-4 ± 5
Q_{O_2}	6.2 ± 0.2	-22 ± 5*	9.7 ± 0.5*	-5 ± 2‡	-23 ± 4*

The data indicate the means$\pm$ . . . of the number of hearts n given in the parentheses. The effect of nifedipine on each parameters is indicated as percentage change from the control value of the parameter obtained without nifedipine under similar conditions. These control values are indicated except for isoprenaline+atenolol. When these control values differed significantly from the control values in WKY rats (Table 2), they are labelled as †($P<0.05$). Statistically significant variations induced by nifedipine or isoprenaline are indicated as * ($P<0.05$). When the effects of nifedipine in isoprenaline differed significantly from its effects under basal conditions, they are indicated as ‡($P<0.01$). The effects of nifedipine in atenolol+isoprenaline were not significantly different from its effects under basal conditions.

by isoprenaline. In fact, as summarized in Table 2, nifedipine applied in the presence of isoprenaline did not induce any significant change in the parameters of contraction or relaxation of WKY rat hearts. Atenolol was subsequently used to antagonize the effect of a β-adrenergic stimulation. The comparison of the contractile traces c and d in Figure 2(b) clearly shows that 1 μ atenolol reduced LVP and increased the time to peak of contraction. In addition, atenolol also reduced work and oxygen consumption, and slowed down contraction and relaxation (Table 2). When applied in the concomitant presence of isoprenaline and atenolol, nifedipine (0.1 μ) had a more pronounced effect on the amplitude of contraction [Fig. 2(a) and traces d and e in Fig. 2(b)]. The effects of nifedipine in the presence of isoprenaline and atenolol on the mechanical and energetic parameters of WKY rat hearts are summarized in Table 2. These effects were not significantly different from those observed previously in the absence of isoprenaline.

Effects of nifedipine in SHR rat hearts

The same experiments as above were performed in SHR rat hearts and are summarized in Table 3. Although the control mechanical parameters were substantially different in both groups of animals, the effects of nifedipine applied under basal conditions were identical. Application of 0.1 μ isoprenaline had a less pronounced effect on LVP in SHR rats than in WKY rats. In SHR rat hearts, LVP peaked ≈ 20 s after application of isoprenaline and increased only by $+10\pm16\%$ ($n=6$), i.e. significantly less than in WKY rats ($P<0.001$). Moreover, at steady state, LVP was smaller in isoprenaline than in control (Table 3). However, in the presence of isoprenaline, all parameters in SHR rat hearts were identical to those measured in WKY rats (*cf.* Table 3 and Table 2). As with WKY rat hearts, nifedipine (0.1 μ) had negligible effects on all parameters measured in SHR rat hearts after application of isoprenaline. Control effects of nifedipine were restored upon addition of 1 μ atenolol to the isoprenaline-containing solution (Table 3). However, like in WKY rat hearts, atenolol alone did not induce any significant change on basal mechanical and energetic parameters in SHR rat hearts (data not shown).

Because nifedipine produced no significant change in the beating frequency in any of the experimental conditions tested, the effects of 0.1 μ nifedipine in both groups of animals can be summarized simply by the variations in LVP. Figure 3 shows the results expressed in percentage variations of LVP in WKY and SHR rat hearts from the control values measured before application of nifedipine. In WKY rat hearts, LVP decreased by $36\pm2\%$ in

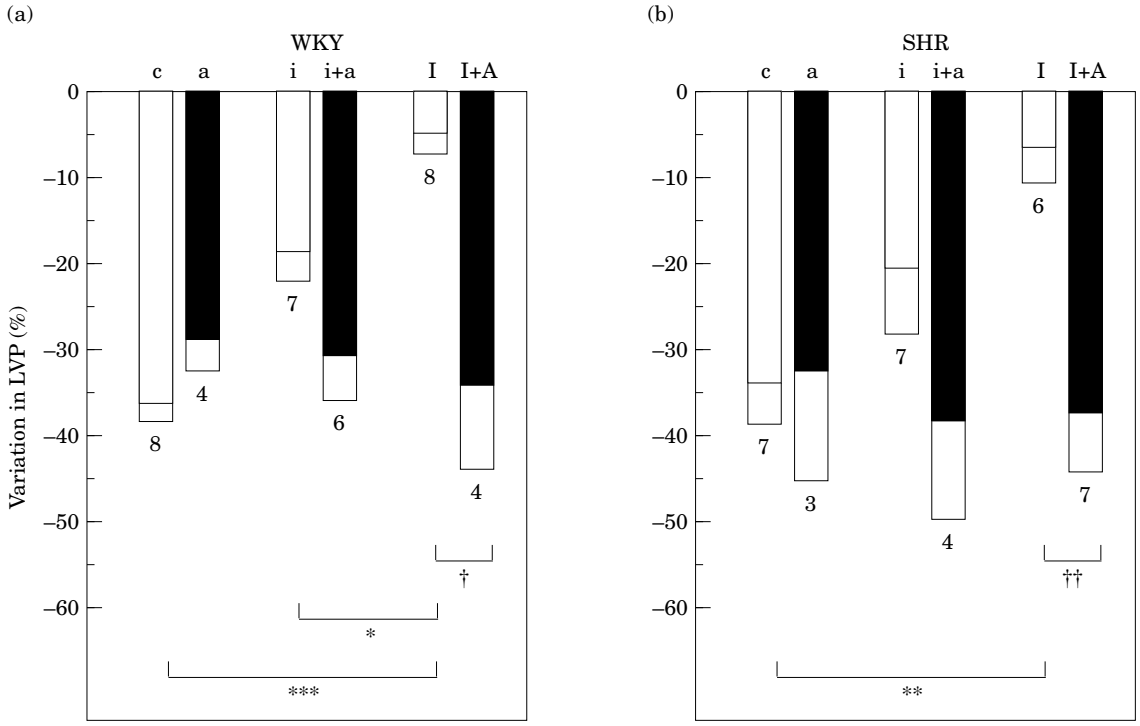

Figure 3 Summary of the effect of nifedipine on LVP in (a) WKY and (b) SHR rats under basal and stimulated conditions. The large bars indicate the means and the small bars the . . . of the results obtained in the number of hearts indicated below the bars. The results are expressed in percentage variation of LVP following the application of nifedipine with respect to the condition prior to the application of the drug. The effects of 0.1 μ nifedipine were tested alone (c, control), or in the presence of 0.1 μ atenolol (a), 0.02 μ isoprenaline (i), 0.02 μ isoprenaline + 0.1 μ atenolol (i + a), 0.1 μ isoprenaline (I), and 0.1 μ isoprenaline + 1 μ atenolol (I + A). The symbols indicate when the presence of isoprenaline (*) or atenolol (†) significantly modified the inhibitory effect of nifedipine on LVP: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; †, $P<0.05$, ††, $P<0.01$. The effects of nifedipine in atenolol (a) and isoprenaline + atenolol (i + a, I + A) were not significantly different from the effect in control (c).

control conditions (c). Addition of increasing concentrations of isoprenaline (0.02 and 0.1 μ) progressively reduced the inhibitory effect of nifedipine on LVP ($18 \pm 3\%$ and $-5 \pm 2\%$ for 0.02 μ (i) and 0.1 μ isoprenaline (I), respectively). Atenolol, used at 0.1 μ with the low (0.02 μ) concentration of isoprenaline (i + a) and at 1 μ with the larger (0.1 μ) concentration (I + A), fully restored the effect of nifedipine to its effect in control. No differences were observed between WKY and SHR rat hearts for the negative inotropic effect of nifedipine measured under all these experimental conditions [Fig. 3(b)].

Effects of nifedipine on Ca current in WKY rat myocytes

Dihydropyridine antagonists block the -type Ca channel activity and decrease I_{Ca} by switching the Ca channel from a conducting mode (mode 1 or 2) to a non-conducting mode (mode 0) (Hess *et al.*, 1984). On the contrary, isoprenaline increases I_{Ca} by recruiting Ca channels from mode 0 to a conducting mode, thus increasing the number of functional channels (Tsien *et al.*, 1986; McDonald *et al.*, 1994). If the mechanisms of Ca channel regulation by nifedipine and isoprenaline were totally independent, one would expect to see no difference in the effect of nifedipine on I_{Ca} under basal and stimulated conditions. In other words, a given concentration of nifedipine should be able to block the same percentage of Ca channels, whether the channels are stimulated by isoprenaline or not. To test this hypothesis, we examined the effect of nifedipine on basal- and isoprenaline-stimulated I_{Ca} in whole-cell patch-clamped WKY rat ventricular myocytes. The cells were depolarized every 8 s to 0 mV during 400 ms from a holding potential of -80 mV. Figure 4(a) shows a typical experiment in which the cell was successively exposed to three concentrations of nifedipine (0.1, 0.3 and 1 μ). First, nifedipine was applied under basal conditions, i.e. in the absence of any stimulation. Nifedipine reduced the peak amplitude of I_{Ca} in a dose-de-

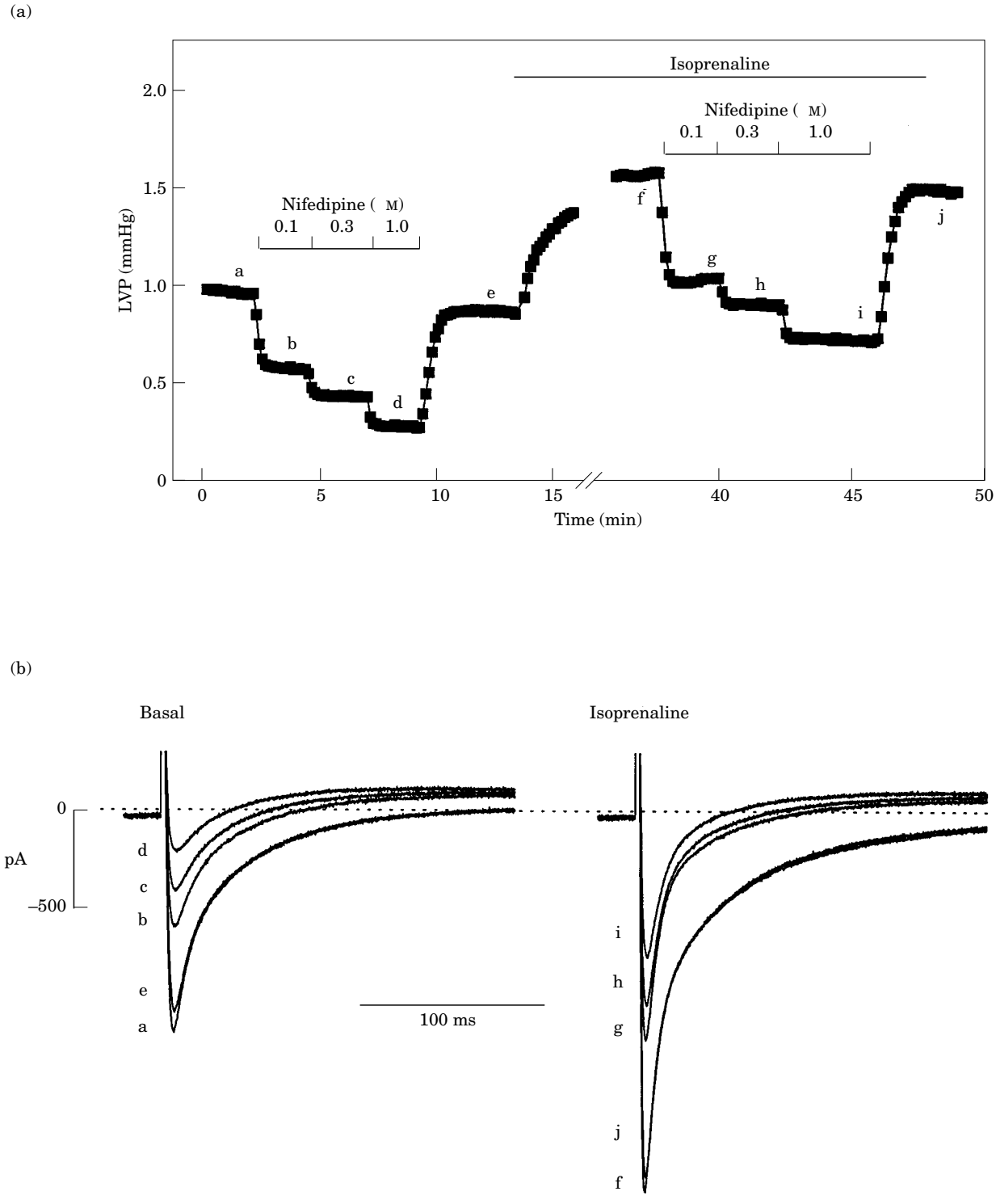

Figure 4 Time course of the effect of nifedipine on basal and stimulated I_{Ca} in a rat ventricular myocyte. (a), I_{Ca} was measured by depolarizing the cell every 8 s to 0 mV during 400 ms from a holding potential of −80 mV. Three increasing concentrations of nifedipine (0.1, 0.3 and 1 μ) were successively applied to the cell during the periods indicated. After wash-out of nifedipine, I_{Ca} recovered its control amplitude. The cell was then exposed to 0.1 μ isoprenaline and again to the same three increasing concentrations of nifedipine, in the continuing presence of isoprenaline. (b) Individual current traces were obtained at the times indicated by the corresponding letters on the graph in (a). Only the first 200 ms of the current obtained during the depolarizing pulse are shown under basal conditions (left) and in the presence of isoprenaline (right). The zero current level is indicated by the dotted line.

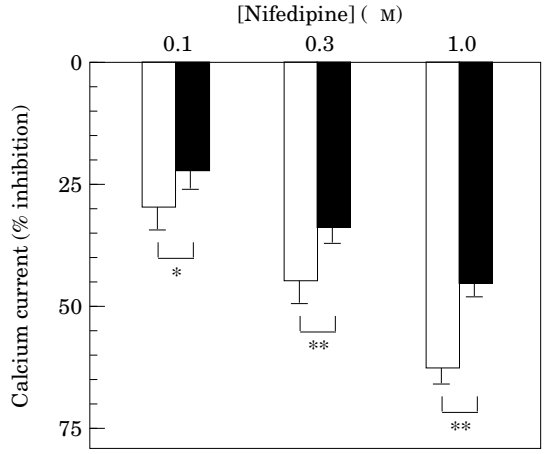

Figure 5 Summary of the effect of nifedipine on I_{Ca} under basal and stimulated conditions. The bargraphs indicate the means and the lines the . . . of the results of four experiments similar to the one shown in Figure 4. The results are expressed as percentage inhibition of I_{Ca} under basal conditions (□) or in the presence of 0.1 μ isoprenaline (■). Statistical differences were obtained using the Student paired *t*-test. * $P<0.05$; ** $P<0.01$.

pendent manner without affecting significantly its kinetics [Fig. 4(b)]. In the presence of 1 μ nifedipine, I_{Ca} was reduced by $\approx$75% to an amplitude of $\approx$250 pA, suggesting that 75% of the total Ca channels were blocked by this concentration of the drug. After wash-out of nifedipine, the current returned to its control level. Exposure of the cell to 0.1 μ isoprenaline produced a large stimulation of I_{Ca}. The same three concentrations of nifedipine were tested again in the presence of isoprenaline. Although nifedipine still reduced peak I_{Ca} in the presence of the β-adrenergic agonist, its effects were substantially smaller than under basal conditions. Indeed, at a concentration of 1 μ , nifedipine reduced I_{Ca} by only $\approx$50% under stimulated conditions to an amplitude of $\approx$750 pA (Fig. 4). Thus, half of the population of Ca channels that were blocked by 1 μ nifedipine under basal conditions were unblocked in the presence of isoprenaline. Figure 5 summarizes the results of four similar experiments. In these experiments, 0.1 μ isoprenaline increased I_{Ca} by 58 $\pm$ 12%. The presence of isoprenaline significantly reduced the inhibitory effect of nifedipine at each of the three concentrations tested. In four other experiments as the one shown in Figure 6, the effect of 1 μ nifedipine was tested successively under basal conditions, in the presence of 0.1 μ isoprenaline, and in the presence of both isoprenaline and 1 μ atenolol. These experiments demonstrated that atenolol, which partially (by 33 $\pm$ 5%) antagonized the stimulatory effect of isoprenaline on I_{Ca}, significantly ($P<0.05$) enhanced the inhibitory effect of nifedipine (Fig. 6). Indeed, in these four experiments, 1 μ nifedipine reduced basal I_{Ca} by 61 $\pm$ 2% under basal conditions, by 46 $\pm$ 4% in the presence of isoprenaline, and by 55 $\pm$ 3% in the concomitant presence of isoprenaline and atenolol.

Discussion

The major finding of these experiments is that activation of β-adrenergic receptors with isoprenaline reduced the inhibitory effect of nifedipine on contraction in the perfused rat heart and attenuated the inhibitory effect of the drug on the -type Ca current of isolated ventricular myocytes. The inhibitory effects of nifedipine were restored upon reduction of the β-adrenergic response with the β1-adrenergic receptor antagonist atenolol.

The a_1 subunit of the -type Ca^{2+} channels possess both the DHP antagonist binding sites and several putative phosphorylation sites (Catterall and Striessnig, 1992; Varadi *et al.*, 1995). In addition, phosphorylation sites are also present on the β subunit, which was shown recently in expression studies to modulate the activity of the channel as well as its sensitivity to DHP agonists and antagonists (Varadi *et al.*, 1995). Therefore, it was conceivable that phosphorylation of the Ca^{2+} channel might modulate the effect of DHPs. However, to our knowledge, relatively few studies have addressed this question specifically. Activation of protein kinase C (Navarro, 1987; Mironneau *et al.*, 1991) or Ca^{2+}-calmodulin-dependent protein kinase (Murphy *et al.*, 1990) was shown to increase the binding of DHP antagonists to voltage-dependent Ca^{2+} channels. In contracting chick muscle cells in culture (Navarro, 1987) and in rabbit heart sarcolemma and skeletal muscle transverse tubules (Murphy *et al.*, 1990) this effect was found to result from an increase in the number of DHP receptors without a significant change in the receptor affinity. However, in membranes of equine portal vein smooth muscle and in intact strips isolated from rat portal vein, activation of protein kinase C enhanced DHP antagonist binding via an increase in the receptor affinity with no change in the receptor number (Mironneau *et al.*, 1991).

Activation of cAMP-dependent protein kinase (cAMP-PK) has led to contrasting results with respect to DHP antagonist binding. In initial studies performed on synaptic membranes from cerebral cortex, a 40% increase in [^{3}H]nitrendipine binding

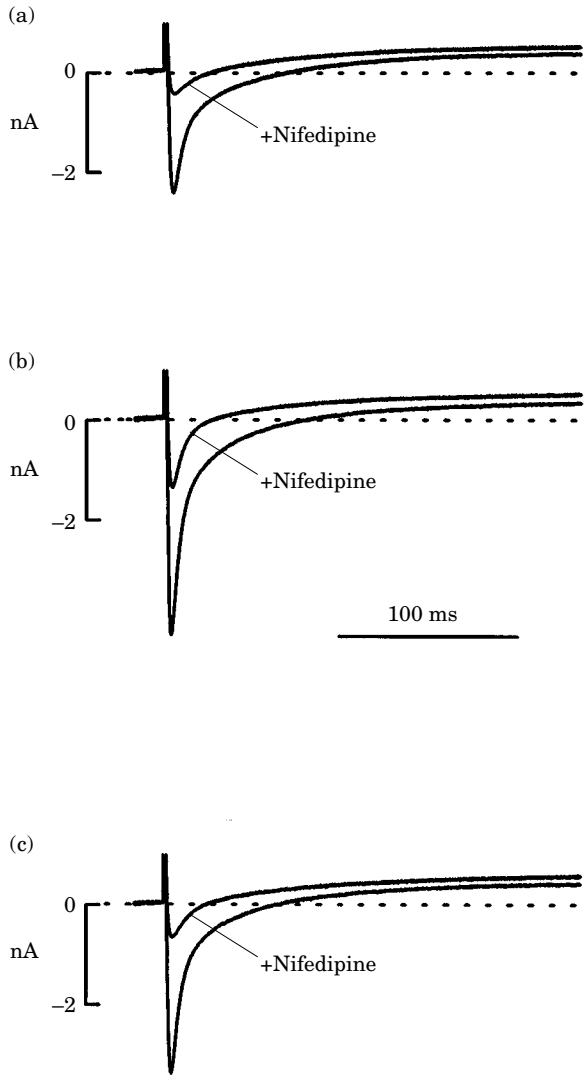

Figure 6 Individual current traces showing the effect of 1 μ nifedipine on I_{Ca} under control conditions (a), in the presence of 0.1 μ isoprenaline (b), and in the presence of isoprenaline + 1 μ atenolol (c). In each set of two traces, the top trace indicates the current in the presence of nifedipine. The dotted lines indicate the zero current level.

was found following activation of cAMP-PK (Wu *et al.*, 1986). However, in rabbit heart sarcolemma and skeletal muscle transverse tubules, no change in [^{3}H]isradipine binding was found following activation of either cAMP-PK or endogenous kinases (Murphy *et al.*, 1990). Apart from these radioligand binding studies, few functional studies have shown that cAMP-dependent phosphorylation modifies the effect of DHPs on isolated cardiac myocytes. In rat ventricular (Tiaho *et al.*, 1990) and in human atrial myocytes (Ouadid *et al.*, 1991), prestimulation of I_{Ca} with either isoprenaline, forskolin or cAMP strongly modified the stimulatory effect of Bay K 8644, a DHP agonist. Indeed, in these preparations, Bay K 8644 which normally accelerated the inactivation of I_{Ca} elicited during a depolarization to either -10 or 0 mV, slowed down dramatically the inactivation after previous stimulation of I_{Ca} by cAMP-PK (Tiaho *et al.*, 1990; Ouadid *et al.*, 1991). Thus, phosphorylated and dephosphorylated -type Ca^{2+} channels appeared to have a distinct pharmacology with respect to DHP agonists. Unfortunately, these authors did not examine the effect of DHP antagonists under similar conditions. However, in a study performed on spontaneously beating chick embryonic ventricular cells, exposure of the cells to isoprenaline was found to shift to the right the entire dose–response curve for the effects of nifedipine on the amplitude of contraction (Briand *et al.*, 1989). This effect seemed to be specific to nifedipine, because the negative inotropic effect of nanomolar (but not micromolar) concentrations of

nicardipine was rather potentiated by isoprenaline (Briand *et al.*, 1989). In another study performed in the rabbit papillary muscle, the negative inotropic effect of several organic calcium antagonists, including nifedipine, was reduced upon stimulation of the contraction with isoprenaline, but not with phenylephrine, an α_1-adrenergic receptor agonist (Kushida *et al.*, 1990).

Although we did not examine the sensitivity of I_{Ca} or contraction to nifedipine in rat heart in detail, our results would tend to confirm the two latter studies (Briand *et al.*, 1989; Kuschida *et al.*, 1990). Indeed, in rat ventricular myocytes, nifedipine had a less pronounced inhibitory effect on I_{Ca} at each of the three concentrations tested (0.1, 0.3 and 1 μ) after the current had been enhanced by isoprenaline (Figs 4 and 5). Moreover, in the isolated perfused heart, the negative inotropic effect of a concentration of 0.1 μ nifedipine was progressively reduced as the concentration of isoprenaline was increased from 0 to 0.1 μ (Fig. 3). In the absence of isoprenaline, the effect of nifedipine, could be essentially summarized by a reduction in LVP accompanied by a slight slowing down of the relaxation phase (Table 2 and 3). Consequent to the reduction in LVP, the index of work (RPP) and the O_2 consumption were reduced accordingly. However, upon stimulation of the hearts with 0.1 μ isoprenaline, these parameters became completely resistant to 0.1 μ nifedipine (Table 2 and 3). This protective effect of isoprenaline appeared to be mediated by activation of β_1-adrenergic receptors, because atenolol (0.1 μ) added to isoprenaline completely restored the effect of nifedipine on all mechanical and energetic parameters (Table 2 and 3). The results obtained with nifedipine, isoprenaline and atenolol on I_{Ca} in isolated ventricular myocytes reproduce at least qualitatively those obtained on contractile activity in isolated perfused hearts. This similarity confirms, if necessary, the general concept that the -type Ca^{2+} current plays a determinant role in the development and amplitude of cardiac contraction (McDonald *et al.*, 1994). This similarity also suggests that phosphorylation of the -type Ca^{2+} channel via activation of cAMP-PK is at least in part responsible for the reduced sensitivity to nifedipine following application of isoprenaline. Indeed, a previous study from our laboratory has demonstrated that the β-adrenergic stimulation of I_{Ca} in rat ventricular myocytes is entirely mediated by activation of cAMP-PK (Hartzell *et al.*, 1991; Hartzell and Fischmeister, 1992). Thus, based on our present study using nifedipine and on previous results using Bay K 8644 (Tiaho *et al.*, 1990; Ouadid *et al.*, 1991), cAMP-dependent phosphorylation likely modifies the conformation of the -type Ca^{2+} channel by changing the binding characteristics of the receptor(s) for DHP agonists and antagonists. It would be interesting to examine whether cAMP-dependent phosphorylation similarly affects the binding of non-dihydropyridine Ca channel antagonists, such as verapamil and diltiazem. However, the reduced sensitivity of I_{Ca} to nifedipine is unlikely to account for all of the protective effect of isoprenaline on the negative inotropic effect of nifedipine. Indeed, we observed a more pronounced effect of isoprenaline on nifedipine inhibition of contractility in the isolated perfused heart than of I_{Ca} in isolated myocytes. This difference may indicate that other mechanisms occurring down stream from sarcolemmal Ca channels may confer to the β-adrenergically stimulated heart a lower sensitivity to a reduction of Ca influx (Kushida *et al.*, 1990).

Under basal conditions, we found that nifedipine had similar effects in WKY and SHR rats (*cf.* Table 2 and 3). However, the protective effect of isoprenaline on nifedipine action was also comparable in WKY and SHR rats (*cf.* Table 2 and 3). This was somewhat surprising because the inotropic response to β-adrenergic stimulation was reduced in SHR rat hearts as compared to WKY (see also Moravec *et al.*, 1995 and refs therein). Although we did not examine this aspect in further detail, our results would tend to confirm the hypothesis that alteration in the β-adrenergic regulation of Ca influx via -type Ca^{2+} channels is not the primary reason for the impaired inotropic response to isoprenaline in SHR rats (Moravec *et al.*, 1995).

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