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Differential potentiation by depolarization of the effects of calcium antagonists on contraction and Ca^{2+} current in guinea-pig heart

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1 The effects of elevation of extracellular K^+ concentration ($[K^+]_o$) on the negative inotropic potencies of three representative calcium antagonists, diltiazem, verapamil and nifedipine, were investigated in guinea-pig papillary muscle preparations.

2 The negative inotropic effect of diltiazem was potentiated 110 fold when $[K^+]_o$ was raised from 2.7 mM to 12.7 mM. The effect of verapamil was also potentiated to a lesser extent, but that of nifedipine was not affected.

3 Resting membrane potentials in ventricular muscles were about − 80 mV and − 60 mV in 2.7 mM K^+ and 12.7 mM K^+, respectively.

4 To clarify the mechanism responsible for the differential potentiation of the negative inotropic effects, the blocking actions of the three calcium antagonists on the L-type Ca^{2+} channel current ($I_{Ca(L)}$) were compared at the holding potentials of − 80 mV and − 60 mV by the whole-cell patch-clamp technique.

5 The use-dependent blocking effect of diltiazem on $I_{Ca(L)}$ was enhanced markedly by the change in the holding potential from − 80 mV to − 60 mV. The effect of verapamil was also enhanced to a lesser extent but that of nifedipine was not affected in this range of depolarization.

6 The differential effects of the $[K^+]_o$ elevation on the negative inotropic potencies of the three calcium antagonists are explained by the differences in voltage-dependency of their use-dependent blocking effects on $I_{Ca(L)}$.

7 The properties of diltiazem and verapamil observed in this study may contribute to their protective effects on the ischaemic myocardium, without affecting the normal myocardium.

Keywords: Diltiazem; verapamil; nifedipine; depolarization; papillary muscle; L-type Ca^{2+} channel

Introduction

Representative calcium antagonists, nifedipine, verapamil and diltiazem are used in the treatment of a wide range of cardiovascular diseases. Although the three agents have been demonstrated to bind to the α_1 subunit of the L-type Ca^{2+} channel (Glossmann & Striessnig, 1990), it is known that they show differences pharmacologically in tissue selectivity.

Evidence has been obtained to indicate that calcium antagonists have beneficial effects on the ischaemic myocardium in models of experimental ischaemia (Nagao *et al.*, 1980; Nakamura *et al.*, 1980; Pérez *et al.*, 1980; Reimer & Jennings, 1985). However, in numerous experimental studies investigating the protective effects of calcium antagonists against myocardial ischaemic damage, the results obtained have been divided into positive and negative, depending on the experimental conditions (Kloner & Braunwald, 1987) and calcium antagonists employed. Ichihara and coworkers reported that diltiazem lessened ischaemic myocardial acidosis evoked by coronary occlusion in the canine heart, and that nifedipine did not (Ichihara & Abiko, 1982; Ichihara *et al.*, 1986). No satisfactory explanation has yet been offered to explain such differences in selectivity of calcium antagonists for the ischaemic myocardium. Not only vasodilator effects but also direct effects on the myocardium, e.g. negative inotropic and negative chronotropic effects, may contribute to the protective effects of calcium antagonists on the ischaemic heart.

The effects of calcium antagonists have been reported to be modulated by stimulus frequency and membrane potential (Hondeghem & Katzung, 1984; Taira, 1987; Triggle, 1991). It has been demonstrated that the extracellular K^+ concentration rises to 10–15 mM in the early phase of ischaemia (Wiegand *et al.*, 1979; Hirche *et al.*, 1980). Thus the potencies of the three calcium antagonists may be affected by the alteration of the membrane potential caused by such elevation of the extracellular K^+ concentration. It is also possible that the voltage-dependency of the effects may differ among the calcium antagonists, which would explain the corresponding differences in their effects on the ischaemic heart. Previous electrophysiological studies have shown that the blocking effects of nifedipine, verapamil and diltiazem on the L-type Ca^{2+} channel current ($I_{Ca(L)}$) are potentiated by depolarization of the membrane potential (Ehara & Kaufmann, 1978; Kanaya & Katzung, 1983; Uehara & Hume, 1985). However, quantitative differences in the voltage-dependency of these drugs have not been well investigated. Thus the differences in voltage-dependency among the calcium antagonists are still unclear.

The present study was designed to examine whether elevation of the extracellular K^+ concentration affects the potencies of the calcium antagonists. For this purpose, we compared the negative inotropic effects of nifedipine, verapamil and diltiazem under normal conditions with those under conditions where the extracellular K^+ concentration was raised to 12.7 mM in guinea-pig papillary muscle preparations. Furthermore, we investigated the relationship between the depolarization caused by elevation of the K^+ concentration and the effects of the calcium antagonists on the L-type Ca^{2+} channel current in single ventricular myocytes.

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Methods

Force measurements in guinea-pig papillary muscles

Male guinea-pigs weighing 150–370 g were anaesthetized with pentobarbitone (40 mg kg^{-1}, i.p.) and killed by cervical dislocation. The whole heart was rapidly removed and immersed in a normal solution of the following composition (mM): NaCl 136.8, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 5.6, $NaHCO_3$ 12, bubbled with 95% O_2 plus 5% CO_2. One papillary muscle was dissected from the left ventricle, and suspended in an organ bath containing the normal solution maintained at 32°C. A resting tension of 0.5 g was applied to the muscle and the preparation was stimulated through a pair of Ag-AgCl electrodes with rectangular pulses (frequency 1 Hz, duration 5 ms, intensity 50% above threshold voltage). The developed tension was measured isometrically by a force transducer. The preparation was allowed to equilibrate for 110 min, the bath solution being changed once at 20 min. Then, the bath solution was exchanged for the normal solution or a high-K^+ solution (KCl concentration 7.7 mM or 12.7 mM). The high-K^+ solution was made by substitution of potassium chloride for the equimolar sodium chloride in the normal solution. Drug administration was started 30 min after this change of the bathing solution. Concentration-response curves were obtained by cumulative application of the drug to the organ bath every 30 min. The potency of the negative inotropic effect was expressed as an IC_{50} value (the concentration of drug required to produce a 50% decrease in the developed tension), which was derived by interpolation of the concentration-response curve.

Measurements of resting membrane potential in ventricular muscles

The whole heart was removed rapidly from male guinea-pigs anaesthetized as described above, and immersed in the normal solution. A small piece of right ventricular muscle was dissected out and fixed in a tissue chamber perfused with the normal solution or the high-K^+ solution at 32°C. An action potential was evoked by rectangular pulses (frequency 1 Hz, duration 5 ms, intensity 50% above threshold voltage).

Resting membrane potentials were recorded via conventional glass micro-electrodes filled with 3 M KCl (resistance 10–20 MΩ). The micro-electrode was coupled via an Ag-AgCl junction to a high-impedance amplifier (MEZ 9201, Nihon Kohden). An Ag-AgCl wire was used as the reference electrode. Data were obtained from the preparations in which the measured resting membrane potentials were stable for over 10 min.

Preparation of single cells

Ventricular myocytes were isolated enzymatically from hearts of male guinea-pigs weighing 200–550 g according to the method described by Cavalié *et al.* (1983). The animals were anaesthetized with pentobarbitone and the ascending aorta was cannulated *in situ* under artificial respiration. The heart was excised and perfused at 37°C by the Langendorff method with collagenase solution for 10–15 min, followed by a high-potassium, Ca^{2+}-free solution (KB solution) for another 10 min. The KB solution (Isenberg & Klockner, 1982) contained (mM): potassium glutamate 70, KCl 25, oxalic acid 10, KH_2PO_4 10, taurine 10, glucose 11, HEPES 10, EGTA 0.5 (pH 7.4, adjusted with KOH). The ventricles were cut into small fragments in KB solution. Ventricular myocytes were dissociated by gentle stirring of the fragments. The dissociated cells were then kept in KB solution at 4°C. The normal Tyrode solution used had the following composition (mM): NaCl 135, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 1.8, glucose 11, HEPES 5 (pH 7.4). The Ca^{2+}-free Tyrode solution had the same composition as the normal Tyrode solution except for the Ca^{2+} concentration, which was nominally zero. The collagenase solution was made up in Ca^{2+}-free Tyrode solution with 100 units ml^{-1} collagenase (Nitta Gelatine) and 18 μM $CaCl_2$.

The cells were placed in a chamber (0.5 ml capacity) attached to the stage of an inverted microscope (IMT-2, Olympus). The chamber was perfused with external solution of the following composition (mM): NaCl 120, KCl 4, $CaCl_2$ 2, $MgCl_2$ 2, glucose 10, HEPES 5, tetraethylammonium chloride 30 (pH 7.4, adjusted with NaOH). All measurements were carried out at room temperature.

Recording of L-type Ca^{2+} channel currents ($I_{Ca(L)}$) in single isolated ventricular cells

$I_{Ca(L)}$s were recorded using the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981). The tip resistance of the fire-polished microelectrodes was 3–4 MΩ when filled with internal solution of the following composition (mM): KCl 80, CsOH 40, tetraethylammonium chloride 20, MgATP 4, EGTA 10, HEPES 10 (pH 7.4, adjusted with HCl). $I_{Ca(L)}$s were elicited by a voltage-clamp step to 10 mV for 200 ms with a 150 ms prepulse to −40 mV to inactivate the voltage-dependent Na^+ channel current and T-type Ca^{2+} channel current. Transmembrane currents were obtained using a DAGAN 8900 amplifier with a 100 MΩ headstage, low-pass filtered at 1 kHz. Data were recorded on a pen recorder (FBR 252-A, TOA) through an oscilloscope (VC-10, Nihon Kohden), stored on videotape via a 16-bit PCM data processor (PCM-DP 16, Shoshin E.M., sampling rate 44.1 kHz), and then analysed using a software programme called 'C-minus' kindly provided by Dr K. Enomoto (Shimane Medical University) with modification by Dr H. Kasai (University of Tokyo). Command pulses were applied using a computer-operated stimulator (Shoshin E.M. Model OI-8).

After formation of a gigaseal and rupture of the membrane, the $I_{Ca(L)}$, which was activated at 0.067 Hz, was allowed to stabilize for 3–5 min at a holding potential (V_h) of −90 mV. Then the frequency of the stimulation was raised to 1 Hz, and approximately 3 min was allowed in order for $I_{Ca(L)}$ to become stable. The external solution with or without the drug was perfused for another 3–5 min. When $I_{Ca(L)}$ became stable, command pulses were stopped and the cell was rested at V_h −90 mV for 30 s. Then V_h was changed to −80 mV, and a further 10 s rest was followed by stimulation at 1 Hz. V_h was kept at −80 mV for 2 min until $I_{Ca(L)}$ reached the new steady state level. Subsequently, the same protocol was repeated at V_h −90 mV and −60 mV. In control experiments, $I_{Ca(L)}$s were measured in the absence of the drug with the same voltage protocol described above. The recovery from block was confirmed by changing V_h from −60 mV to −90 mV to assess the degree of rundown of $I_{Ca(L)}$. Data showing marked rundown were omitted. At the end of the experiment, leakage current was measured in the presence of 0.2 mM $CdCl_2$, which was subtracted from the recorded current to obtain the peak $I_{Ca(L)}$ amplitude. The peak $I_{Ca(L)}$ amplitude was normalized with the $I_{Ca(L)}$ amplitude at V_h −90 mV immediately before drug application as 100%.

Drugs used

The following drugs were used: (±)-verapamil.HCl (Nacalai Tesque), nifedipine (Sigma). (+)-*cis*-diltiazem was kindly supplied by Tanabe Seiyaku Co.Ltd., Saitama, Japan. Diltiazem and verapamil were dissolved and diluted in distilled water. Nifedipine was dissolved in absolute ethanol at a concentration of 10^{-3} M. Further dilutions of nifedipine were prepared in distilled water. Because of the light-sensitive nature of nifedipine, all experiments were carried out under subdued lighting and the solutions were protected from light.

Statistical analysis

Bonferroni's multiple t test or Student's t test was employed to assess the statistical significance of differences. Differences at $P<0.05$ were considered to be significant. All data in the figures, tables and text are represented as the mean ± s.e. mean.

Results

Effect of extracellular K^+ concentration on the negative inotropic potencies of the calcium antagonists in papillary muscle preparations

Elevation of the extracellular K^+ concentration from 2.7 mM to 12.7 mM reduced the developed tension of papillary muscles to 66.1 ± 6.9% ($n = 6$) and 73.8 ± 6.3% ($n = 18$), respectively, taking the developed tension measured in the normal solution just before the change of the bath solution as 100%. When the bath solution was exchanged with the normal solution, the developed tension did not alter (97.8 ± 2.0%, $n = 18$).

Developed tension of papillary muscles decreased gradually in a time-dependent manner under normal conditions and in the presence of 12.7 mM K^+ in the absence of drugs. When the preparation was treated with vehicle instead of drugs, the developed tension was reduced to about 70% of the initial value 150 min after the first administration of vehicle under both conditions (data not shown). However, the degree of reduction of the developed tension did not differ between the normal and 12.7 mM K^+ conditions, and therefore the time-dependent decrease in the developed tension was not corrected.

Figure 1 shows the concentration-response curves for the negative inotropic effects of diltiazem, verapamil and nifedipine under normal and high-K^+ conditions. The three drugs caused concentration-dependent decreases in developed tension under normal and high-K^+ conditions (Figure 1). The concentration-response curve for diltiazem was shifted markedly to the left by the elevation of extracellular K^+ concentration. Although the concentration-response curve for verapamil was shifted leftward in the presence of 12.7 mM K^+, the degree of potentiation was less than that for diltiazem. The concentration-response curve for nifedipine was unaffected by the elevation of K^+ concentration. IC_{50} values for each drug obtained under the respective conditions, calculated from the concentration-response curves, are shown in Table 1. The rank order of potency of the negative inotropic effect obtained under normal conditions was nifedipine > verapamil > diltiazem. The IC_{50} value for each drug under normal conditions was divided by the IC_{50} value under high-K^+ conditions to obtain the IC_{50} ratio (Table 1), which represents the degree of potentiation of the negative inotropic effects of the drugs caused by the increase of extracellular K^+ concentration. The rank order of the degree of the potentiation was diltiazem > verapamil > nifedipine.

Measurements of resting membrane potentials

Resting membrane potentials in ventricular muscles were measured under normal and high-K^+ conditions. Normal action potentials were evoked by electrical stimulation in each preparation. The mean resting membrane potentials under normal conditions, in the presence of 7.7 mM K^+ and 12.7 mM K^+ were − 82 ± 1 mV, − 67 ± 1 mV and − 59 ± 1 mV, respectively (mean ± s.e.mean of 10 experiments). The results were close to the values reported by other investigators (Nakajima *et al.*, 1976).

In the subsequent $I_{Ca(L)}$ measurements, holding potentials of − 80 mV and − 60 mV were used as the conditions corresponding to normal and 12.7 mM K^+ conditions, respectively, in the papillary muscle preparations.

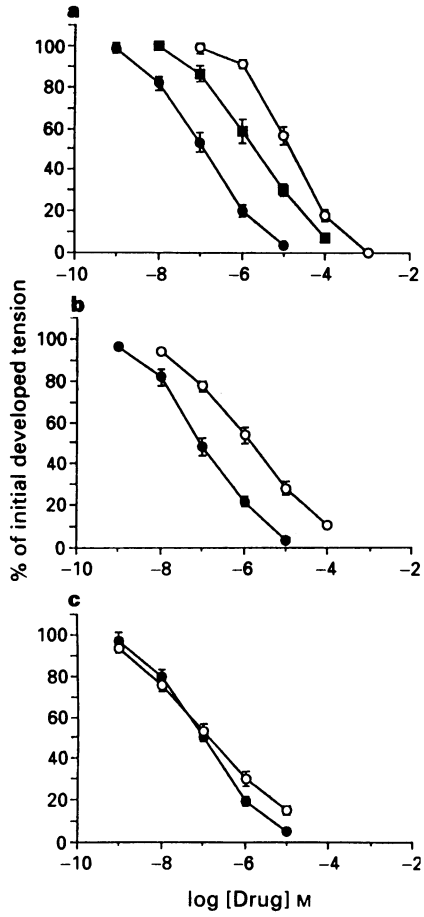

Figure 1 Negative inotropic effects of (a) diltiazem, (b) verapamil and (c) nifedipine on guinea-pig papillary muscles under normal conditions (○) and in 7.7 mM K^+ (■) and 12.7 mM K^+ (●).

Effect of depolarization on the $I_{Ca(L)}$-blocking action of calcium antagonists in single ventricular myocytes

Figure 2 shows typical time courses of $I_{Ca(L)}$ under control conditions (Figure 2a), and in the presence of diltiazem at 3×10^{-6} M (Figure 2b), verapamil at 3×10^{-7} M (Figure 2c) and nifedipine at 3×10^{-8} M (Figure 2d). The drug concentration chosen was that at which the drug suppressed the amplitude of $I_{Ca(L)}$ to about 60% at V_h − 80 mV at a frequency of 1 Hz. Each drug began to block $I_{Ca(L)}$ rapidly after administration, and the effect reached a steady-state within a few minutes. After the change in V_h, stabilization for 2 min allowed the amplitude of $I_{Ca(L)}$ to reach a new steady-state level. In the right-hand panel of Figure 2, the $I_{Ca(L)}$ traces recorded at the times indicated by the arrows in the left-hand panel, at V_h − 60 mV and − 80 mV, are superimposed. Under control conditions, there was little difference between the amplitude of $I_{Ca(L)}$ at V_h − 80 mV and that at V_h − 60 mV (Figure 2a). The blocking effects of diltiazem and verapamil were augmented in a use-dependent manner. In contrast, blockade of $I_{Ca(L)}$ by 3×10^{-6} M diltiazem was markedly enhanced by changing V_h from − 80 mV to − 60 mV (Figure 2b). The blocking effect of 3×10^{-7} M verapamil was also enhanced by the depolarization, but the degree of enhancement was less than that for diltiazem (Figure 2c). The effect of 3×10^{-8} M nifedipine was enhanced only slightly by the depolarization (Figure 2d).

Figure 3 summarizes the amplitudes of $I_{Ca(L)}$ elicited by the first test pulse applied at V_h − 80 mV and − 60 mV after a resting period of 10 s in the presence and absence of drug. None of the calcium antagonists induced any significant tonic block at V_h − 80 mV and − 60 mV.

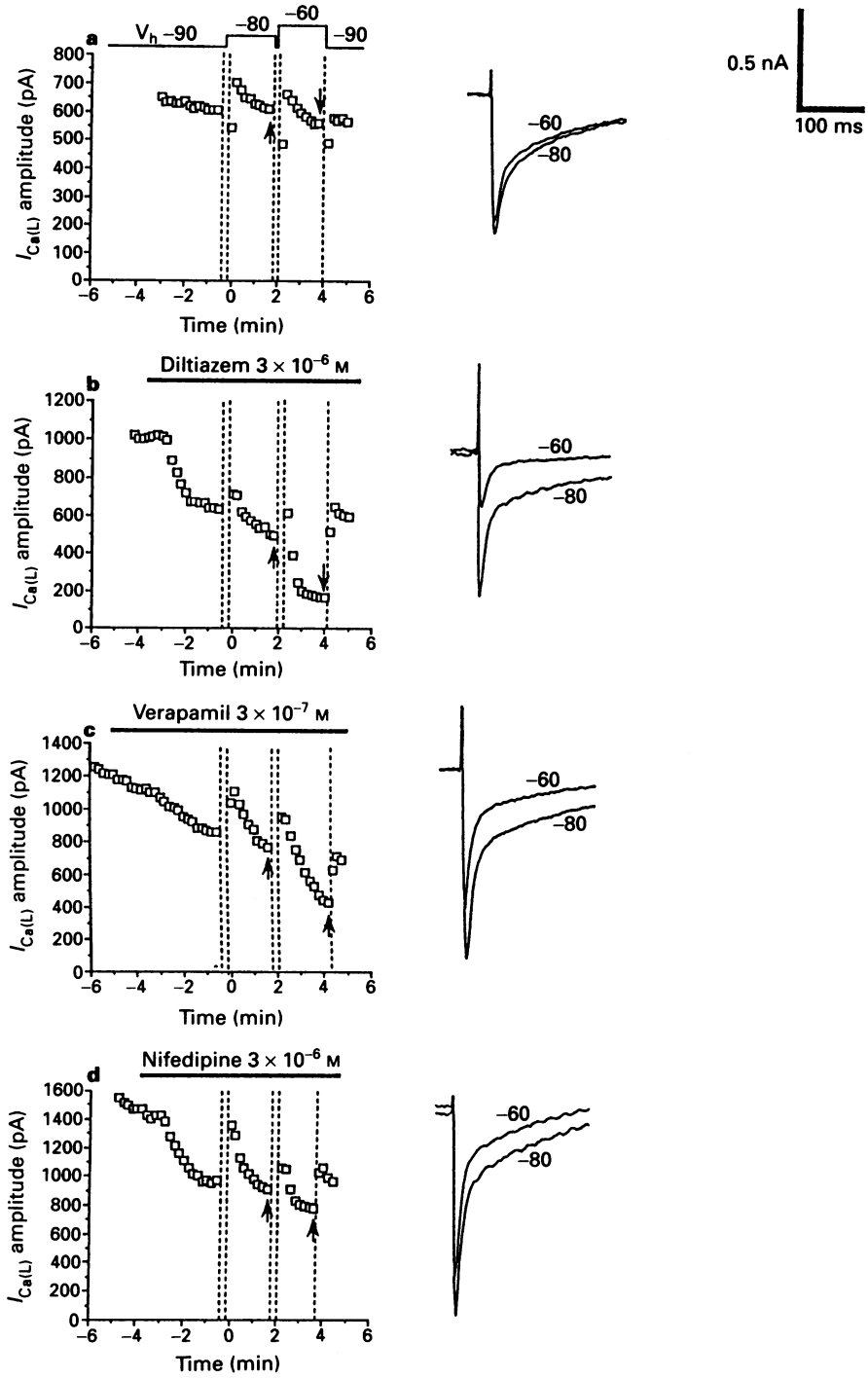

Figure 2 Differential voltage-dependent block of $I_{Ca(L)}$ by the three calcium antagonists. Left panels show typical time courses of peak $I_{Ca(L)}$. Every 12th record of the peak $I_{Ca(L)}$ was plotted. The recordings were made in the normal external solution (a) and in the normal external solution containing (b) 3×10^{-6} M diltiazem, (c) 3×10^{-7} M verapamil, (d) 3×10^{-8} M nifedipine during the period indicated by the horizontal lines. Holding potential (V_h) was changed in turn from -90 to -80, -60 to -90 mV as indicated in (a). During the period indicated by the vertical dashed lines, command pulses were stopped. In the right-hand panel, the superimposed current traces recorded at the times indicated by arrows in the left panels at V_h -80 mV (-80) and V_h -60 mV (-60) are shown.

Figure 4 shows the amplitude of $I_{Ca(L)}$ at the steady-state level about 2 min after the change in V_h. The blocking effects on the amplitude of $I_{Ca(L)}$ shown in Figure 4 represent the sum of tonic blockade and the block accelerated by repetitive channel opening, i.e. 'use-dependent block'. Under control conditions, the amplitude of $I_{Ca(L)}$ was little changed when V_h was changed from -80 mV to -60 mV. In contrast, the blocking effect of diltiazem was markedly enhanced when V_h was depolarized. The effect of verapamil was enhanced significantly, but the degree of enhancement was less than that for diltiazem. The effect of nifedipine was not affected significantly by V_h depolarization.

Discussion

We compared quantitatively the effect of elevation of extracellular K^+ concentration on the negative inotropic effects of three calcium antagonists. In the present study, we focused on the differential modulation of the effects of calcium

Table 1 Effects of elevation of extracellular K^+ concentration upon the negative inotropic potencies of diltiazem, verapamil and nifedipine

	IC_{50} (μM) (pIC_{50})			IC_{50} ratio	
Drug	*Normal*	7.7 mM K^+	12.7 mM K^+	*Normal* / 7.7 mM K^+	*Normal* / 12.7 mM K^+
Diltiazem	16.1 ± 0.41	2.27 ± 0.62	0.145 ± 0.036	7.1	110
	(4.85 ± 0.10)	(5.76 ± 0.15)*	(6.92 ± 0.12)*		
Verapamil	1.93 ± 0.84		0.112 ± 0.043		17
	(5.86 ± 0.15)		(7.05 ± 0.12)†		
Nifedipine	0.185 ± 0.052		0.110 ± 0.016		1.7
	(6.86 ± 0.16)		(6.99 ± 0.06)^{NS}		

IC_{50} (pIC_{50}) values are the mean ± s.e.mean for six experiments. IC_{50} ratio values represent the ratio of normal: 7.7 mM K^+ or normal: 12.7 mM K^+ mean IC_{50}. Individual pIC_{50} values for each drug under high-K^+ conditions were compared statistically with the corresponding pIC_{50} values obtained under normal conditions.
NS = not significantly different; *$P<0.001$, Bonferroni's multiple t test.
†$P<0.001$, Student's t test.

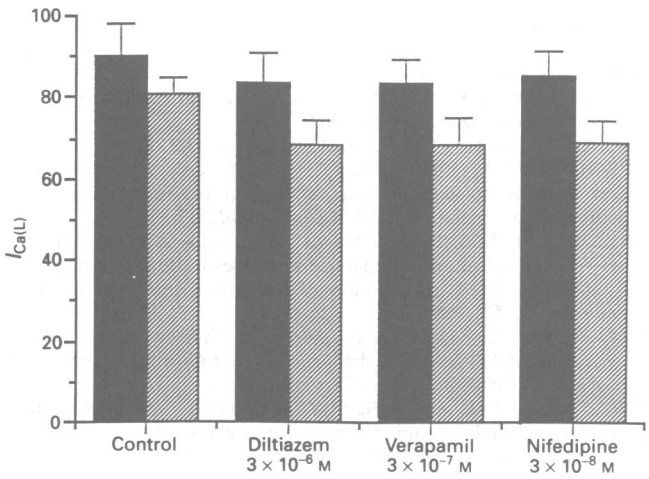

Figure 3 Tonic inhibition of $I_{Ca(L)}$ by 3×10^{-6} M diltiazem, 3×10^{-7} M verapamil and 3×10^{-8} M nifedipine at V_h − 80 mV (solid columns) and V_h − 60 mV (hatched columns). Values are means with s.e.mean for six experiments.

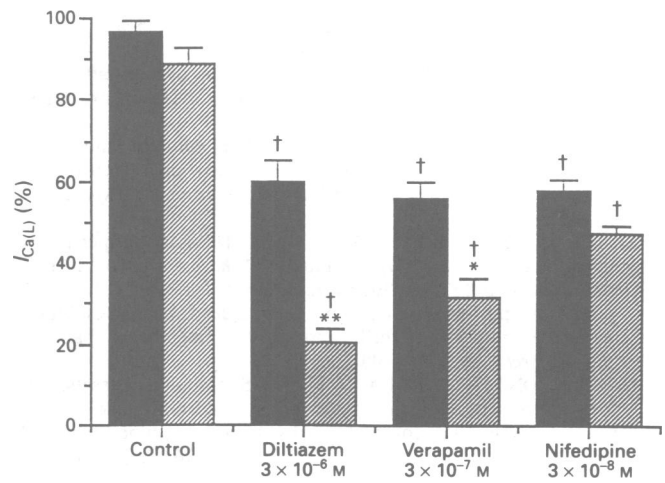

Figure 4 Inhibition of peak $I_{Ca(L)}$ activated at 1 Hz by 3×10^{-6} M diltiazem, 3×10^{-7} M verapamil and 3×10^{-8} M nifedipine at V_h − 80 mV (solid columns) and V_h − 60 mV (hatched columns). Values are means with s.e.mean for six experiments. †$P<0.001$ vs control; *$P<0.01$; **$P<0.001$ vs V_h − 80 mV.

antagonists by the membrane depolarization resulting from elevation of the extracellular K^+ concentration.

One of the most important findings of this study was that the degree of potentiation of the negative inotropic effects resulting from elevation of the extracellular K^+ concentration varied among diltiazem, verapamil and nifedipine.

The recording of $I_{Ca(L)}$ made it clear that the $I_{Ca(L)}$-blocking actions of the three calcium antagonists were differentially augmented by changing V_h from − 80 mV to − 60 mV, corresponding to the respective membrane potentials of the ventricular myocytes under normal and 12.7 mM K^+ conditions. The rank order of augmentation of the $I_{Ca(L)}$-blocking action among the three calcium antagonists agreed well with the rank order of potentiation of their negative inotropic effects induced by elevation of the extracellular K^+ concentration. These results suggest that the differences in voltage-dependency of the $I_{Ca(L)}$-blocking action may be responsible for the differences in potentiation of the negative inotropic effects among the calcium antagonists.

In the recording of $I_{Ca(L)}$, the concentration of each drug was chosen so as to reduce the amplitude of $I_{Ca(L)}$ to approximately 60% at V_h − 80 mV at a frequency of 1 Hz (see Figure 2). The concentration of nifedipine was the lowest; that of verapamil was 10 fold and that of diltiazem was 100 fold higher than that of nifedipine. This concentration ratio was consistent with the relative potencies of the negative inotropic effects of the three drugs under normal conditions in papillary muscles.

In the present study, the component of the $I_{Ca(L)}$-blocking action of the three calcium antagonists that was enhanced by the membrane depolarization was the use-dependent block.

In the present study, nifedipine showed use-dependent block without significant tonic block of $I_{Ca(L)}$, although dihydropyridine has been reported to produce marked tonic block (Lee & Tsien, 1983). One of the possible reasons for this was that nifedipine at 3×10^{-8} M was at a rather low concentration to produce detectable tonic block. Another explanation may be that nifedipine also shows use-dependent block when a stimulus frequency higher than 1 Hz is applied (Uehara & Hume, 1985).

The present findings suggest that the negative inotropic effects of diltiazem and verapamil may be potentiated by an increase of extracellular K^+ concentration under certain ischaemic conditions. Drugs which reduce myocardial contraction locally and decrease oxygen demand are desirable as therapeutic agents for myocardial ischaemia. However, nifedipine, verapamil and diltiazem are known to be far more potent in producing coronary vasodilatation than in producing negative inotropic effects. Himori *et al.* (1976) reported that the doses of diltiazem and verapamil producing a 50% decrease in the developed tension were 5.2 and 3.5 times higher than the doses producing a 100% increase in blood

flow rate, respectively, in canine isolated blood-perfused papillary muscles. Nevertheless, the present results suggest that dilitiazem and verapamil, at doses which produce coronary vasodilatation, can reduce contractile force when extracellular K^+ concentration is elevated. In the case of regional ischaemia, these drugs may preferentially suppress contraction of the ischaemic area at doses producing no effects on normal myocardium. This may partly explain the differential selectivity of the calcium antagonists in the ischaemic heart (see Introduction).

Under ischaemic conditions, various metabolic and ionic changes such as milieu pH changes are known to occur other than elevation of extracellular K^+ concentration. These changes may also affect the potencies of calcium antagonists. Recently, calcium antagonists have been reported to inhibit the mitochondrial Na-Ca antiporter (Cox & Matlib, 1993) and anion channel (Zernig, 1990). Therefore effects of these drugs on mitochondria cannot be disregarded.

In conclusion, the differential potentiation of the negative inotropic effects of the three calcium antagonists by an increase in extracellular K^+ concentration can be explained by the difference in voltage-dependency of the use-dependent block of $I_{Ca(L)}$. The voltage-dependency of the use-dependent effects of diltiazem and verapamil may contribute to their protective effects on the ischaemic myocardium, without affecting the normal myocardium.

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