Effects of hypoxia, elevated K^+ and acidosis on the potency of verapamil, diltiazem and nifedipine in the guinea-pig isolated papillary muscle

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1 The effects of the structurally diverse calcium channel blockers verapamil, nifedipine and diltiazem were investigated on the force of contraction of guinea-pig, electrically stimulated papillary muscles *in vitro*.

2 Calcium channel blocking potency was assessed either as a direct negative inotropic effect or as the ability of the drugs to antagonise the positive inotropic effects of added calcium (Ca^{2+}). By either method, the rank order of potency was found to be nifedipine > verapamil > diltiazem.

3 Various factors which mimic some of the consequences of acute ischaemia *in vivo*, namely low pH, hypoxia and elevated K^+ , in combination, but not singly, enhanced the negative inotropic potency of verapamil and to lesser extent that of diltiazem, but not that of nifedipine.

4 Whilst the various interventions, especially in combination, produced a profound negative inotropic effect themselves, this was not responsible *per se* for the potentiation of the negative inotropic effects of verapamil and diltiazem, since the negative inotropic effects of nifedipine and dinitrophenol were not potentiated under the 'ischaemic' conditions.

5 By use of antagonism of the positive inotropic action of exogenous Ca^{2+} as an alternative measure of potency, the differential influence of 'ischaemia' on calcium channel blocker potency was confirmed. The effect of verapamil was potentiated some 9 fold, that of diltiazem about 2 fold, and that of nifedipine was unchanged.

6 The differential effect of 'ischaemia' on the potencies of the calcium channel blockers was unexpected. Verapamil (but not nifedipine) is thought to bind to the calcium channel at a specific site not easily accessible from the extracellular space. 'Ischaemic' conditions may cause membranous perturbations which allow verapamil easier access to its binding site thus increasing its negative inotropic potency.

Introduction

The calcium channel blocking drugs which comprise several structurally diverse classes of drugs are potent modulators of the functional activity of vascular smooth muscle and cardiac muscle. Their therapeutic usefulness has been proven in the treatment of a wide range of cardiovascular diseases which include hypertension (Buhler & Kiowski, 1988), angina (Hugenholz, 1988) and supraventricular tachycardia (see Singh, 1986). This therapeutic activity largely arises from the vasodilator and negative inotropic, negative dromotropic and negative chronotropic properties of the compounds.

Several studies have provided evidence that the

potency of certain calcium channel blocking drugs as negative inotropic agents is enhanced in the ischaemic, compared with the normally perfused, myocardium of the anaesthetized dog (Smith *et al.*, 1976; Abrahamsson *et al.*, 1985). However, analysis of the direct cardiac action of drugs *in vivo* can be complicated by systemic effects of these same agents. Thus, for example, profound vasodilatation produced by calcium channel blocking drugs could influence myocardial contractility indirectly to varying degrees depending on the calcium channel blocker used. In an attempt to circumvent this problem Smith and coworkers (Briscoe & Smith, 1982; Smith & Briscoe, 1985) examined the calcium antagonistic potencies of several calcium channel blockers *in vitro* on cat, electrically stimulated papillary muscle both in a normal

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physiological salt solution and in one with an artificially lowered pH. With verapamil, for example, an 8 fold increase in Ca^{2+} antagonistic potency was observed under the acidotic conditions. Other studies have shown a potentiating effect of elevated extracellular K^+ upon calcium channel blocking potency in rat isolated perfused hearts (Curtis & Walker, 1986; 1988). However, acidosis and elevated K^+ represent only two of several cellular consequences of ischaemia (see Gettes, 1986), the other obvious consequence being hypoxia. If acidosis and elevated extracellular K^+ alone potentiate the negative inotropic potency of calcium channel blocking drugs, it is possible that a combination of these factors plus hypoxia may have a more profound potentiating effect and one more closely resembling that occurring during ischaemia *in vivo*.

In the present study it was therefore our aim to examine the effect of these various factors which occur during ischaemia *in vivo*, alone and in combination, upon the potency of calcium channel blocking drugs *in vitro*. To this end, the calcium channel blocking potencies of verapamil, diltiazem and nifedipine were assessed in guinea-pig isolated, electrically stimulated papillary muscles, both by measuring their direct negative inotropic effects in the presence of a fixed Ca^{2+} concentration and also by measuring the abilities of the drugs to antagonise the positive inotropic effect of exogenously administered Ca^{2+} .

Methods

Guinea-pig isolated papillary muscle preparation

Male guinea-pigs weighing between 250 g and 400 g were killed by cervical dislocation. The whole heart was rapidly removed and placed in a Petri dish containing a physiological salt solution of the following composition (mm): NaCl 118, NaHCO_3 25, KCl 4.7, MgSO_4 0.6, KH_2PO_4 1.2, glucose 11, CaCl_2 1.3, vigorously bubbled with 95% O_2 plus 5% CO_2 . Two papillary muscles were carefully dissected from the right ventricle. Individual papillary muscles were suspended vertically between platinum stimulating electrodes in glass tissue chambers (20 ml capacity) and each connected by a cotton thread to a force transducer (Dynamometer UF1). The tissue chambers contained physiological salt solution bubbled with 95% O_2 plus 5% CO_2 and maintained at 32°C. It has been shown previously (see Furchgott, 1972) that the viability of electrically stimulated papillary muscles is better maintained at this temperature than at 37°C. A resting tension of 0.5 g was applied to each muscle and the preparations were individually stimulated at a frequency of 1 Hz at 5 V, with square

wave pulses of 5 ms duration (Glaxo Multi-Range Stimulator) during a 1 h equilibration period. Changes in developed tension were displayed on an 8-channel pen recorder (Ormed MX8). Readjustment of any deviation from the resting tension was made only during the equilibration period. Following the equilibration period, the voltage was reduced to zero and then gradually increased until full contraction of the preparation was elicited and then further increased by 10% (threshold voltage; usually approximately 1 V). The frequency of stimulation was also increased to 3 Hz. It was rarely necessary in an experiment to alter the stimulation voltage, although on some occasions if contraction failed (especially when external Ca^{2+} concentration was raised above 10 mM; see below) it was gradually increased until mechanical activity was fully restored. Following a further second equilibration period of 20 min the basal developed tension of the preparations was 144 ± 8 mg ($n = 138$). The pH, PO_2 and PCO_2 in a sample of the physiological salt solution from each chamber were also measured at this time by an ABL1-Acid Base Laboratory (Radiometer).

Experimental design—negative inotropic potency

In the first series of experiments, following the second equilibration period, one papillary muscle of each pair was exposed to one of the following drugs; verapamil (10 nM–10 μM), nifedipine (10 nM–10 μM), diltiazem (100 nM–10 μM) or dinitrophenol (0.1–10 μM). These were added cumulatively, usually in 3 fold increments in concentration, 30 min contact being allowed at each new concentration. The other papillary muscle of each pair received vehicle alone (control preparation) and was used only to correct the negative inotropic effects obtained from the preparation treated with each test drug, for any spontaneous change in developed tension which occurred over the course of drug administration. Developed tension in each preparation 30 min after the addition of each new concentration of drug was expressed as a percentage of the value immediately before drug addition and the following correction applied:—

Drug-induced effect in drug treated preparation

$$\begin{aligned} & \text{\% developed tension remaining in test} \\ & \quad \text{preparation 30 min after drug} \\ & = \frac{\text{\% developed tension remaining in control}}{\text{preparation 30 min after vehicle}} \times 100 \end{aligned}$$

The corrected data for each drug-treated papillary muscle were plotted as percentage developed tension against concentration of calcium channel blocker. Negative inotropic potency was expressed as an IC_{50} value (the concentration of drug required to produce

a 50% decrease in developed tension), derived by an interpolation from this plot.

In vitro 'ischaemic' conditions

The negative inotropic potency of verapamil was also tested under various conditions *in vitro* which mimic some of the consequences of ischaemia *in vivo*. Following the initial 1 h equilibration period the frequency of stimulation was increased to 3 Hz and voltage reduced to threshold (see above). Five minutes later the various interventions were applied either singly or in various combinations.

Partial depolarisation Extracellular K^+ concentration was raised from 5.9 to 9.2 mM by the addition of KCl to the physiological salt solution, substituted for NaCl so as to maintain osmolarity.

Acidosis pH was reduced from 7.4 to either 6.9 or 6.5 by reducing the $NaHCO_3$ concentration from 25 mM to 8 mM or 3 mM respectively (metabolic acidosis). NaCl concentration was adjusted accordingly to keep total Na^+ concentration constant. In other experiments (respiratory acidosis), pH was lowered to between 6.9 and 7.0 by bubbling with a gas mixture with increased CO_2 content (see below). In each case the pH fell, as dictated by the Henderson-Hasselbach equation.

Hypoxia Relatively hypoxic conditions were created by substituting the usual 95% O_2 /5% CO_2 gas mixture for one comprising 55% O_2 /40% N_2 /5% CO_2 . The CO_2 component was deliberately left unchanged so as not to interfere with pH. The one exception to this was the gas mixture for respiratory acidosis which contained 17.5% CO_2 as well as reduced O_2 (55%) with 27.5% N_2 .

When the combination of 'ischaemic' conditions was applied, the physiological salt solution was changed first (metabolic acidosis, elevated K^+) followed 10 min later by the change in gas mixture (hypoxia). After application of these 'ischaemic' variables (which took up to 20 min in total and caused negative inotropic effects in their own right), a further equilibration period of 20 min was allowed before the first concentration of drug was added.

In further experiments, the negative inotropic potency of verapamil was measured in preparations electrically stimulated at (i) threshold voltage in the presence of propranolol (300 nM) which was present in the bathing solution throughout the entire experiment or (ii) supramaximal voltage (5 V) in the absence or presence of propranolol (300 nM). Initial experiments carried out with supramaximal voltage (5 V) in the absence of propranolol were abandoned

due to marked and variable fade in the developed tension. A protocol was therefore devised in which voltage of stimulation was increased from threshold to 5 V for 5 min and then returned to threshold voltage. This increase in voltage caused a positive inotropic effect and the peak of this response was reproducible if the periods of high voltage stimulation were spaced at intervals of 30 min.

Following two control responses verapamil (or the appropriate amount of vehicle) was added in increasing concentration (10 nM–10 μ M). The first concentration of verapamil was added to the preparation 5 min after the second control voltage increase and left to equilibrate for 25 min. At the end of this time the voltage was increased again and the effect of verapamil on the peak response to the voltage increase measured. The voltage was then reduced, the next concentration of verapamil added, and the procedure repeated. No change of the bathing medium was made at any time after addition of the first concentration of verapamil or vehicle.

Experimental design—antagonism of Ca^{2+} -induced positive inotropic effects

In further experiments, the potencies of the calcium channel blockers were assessed in an alternative way by measuring their abilities to displace Ca^{2+} concentration-response curves, which were obtained when cumulatively increasing concentrations of Ca^{2+} were added sequentially to the preparations. In each experiment four papillary muscles (from 2 or 3 animals) were prepared as before and following the equilibration period the frequency of stimulation was again increased to 3 Hz and the voltage reduced to threshold. The physiological salt solution was then replaced by one containing a reduced concentration of Ca^{2+} (0.6 mM) and after 20 min a cumulative Ca^{2+} concentration-response curve (1.0–9 mM) was constructed on each preparation and the resulting increase in developed tension recorded. The bathing medium was then replaced every 5 min for at least 30 min until the developed tension had returned to the control value. After a further 30 min a second cumulative Ca^{2+} concentration-effect curve was constructed. Following this curve, three of the preparations were exposed to a different concentration of calcium channel blocking drug whilst the fourth preparation was exposed to vehicle alone. Thirty minutes later a third Ca^{2+} concentration-effect curve was constructed. For each individual preparation, total developed tension at each new Ca^{2+} concentration in the third concentration-effect curve was expressed as a percentage of the response to 9 mM Ca^{2+} in the second Ca^{2+} curve and results plotted graphically. Ca^{2+} concentration-effect curves in the presence of the calcium channel blocking drugs were

Table 1 The effect of the ischaemic interventions alone and in combination upon developed tension in guinea-pig papillary muscles

	pH	Po ₂ (mmHg)	Pco ₂ (mmHg)	% change in developed tension	% recovery of initial developed tension in verapamil-untreated preparations ^a
A Normal (n = 68)	7.4 ±0.004	560 ±4	35 ±0.2	3 ± 2	—
B Mild metabolic acidosis (n = 10)	6.9 ±0.006	526 ±6	33 ±1.4	5 ± 10	133 ± 22
C Severe metabolic acidosis (n = 10)	6.5 ±0.01	543 ±5	32 ±0.3	-50 ± 8	118 ± 8
D Hypoxia (n = 12)	7.4 ±0.003	354 ±3	36 ±0.3	-39 ± 4	83 ± 9
E 9.2 mM K ⁺ (n = 8)	7.4 ±0.004	580 ±7	36 ±0.7	-30 ± 5	78 ± 10
F Mild metabolic acidosis + hypoxia (n = 10)	6.9 ±0.004	352 ±4	35 ±0.2	-50 ± 3	107 ± 12
G Mild metabolic acidosis + hypoxia + 9.2 mM K ⁺ (‘ischaemia’) (n = 20)	6.9 ±0.007	341 ±3	34 ±0.4	-70 ± 2	109 ± 8
H Mild respiratory acidosis + hypoxia + 9.2 mM K ⁺ (n = 14)	7.0 ±0.01	288 ±13	91 ±5.0	-88 ± 1	55 ± 6

^a Values were recorded 20 min after return to normal conditions and represent values from only half of the preparations studied (i.e. those receiving vehicle).

usually parallel with the control curve. However, in some experiments the curves became non-parallel above the 50% response level. Responses were therefore compared, at the EC₄₀ level where the curves were consistently parallel. Ca²⁺ concentration-ratios (CR) were calculated for each concentration of calcium channel blocker or its vehicle by dividing the EC₄₀ value obtained in the presence of test drug or vehicle (third Ca²⁺ curve) by the EC₄₀ value obtained from the corresponding curve obtained in the absence of drug (second Ca²⁺ curve). The CR values from the drug-treated preparations were divided by the CR value from the vehicle-treated preparation to correct for any spontaneous changes in sensitivity. For each experiment drug potency was then expressed graphically by plotting the log₁₀ of these corrected CR values against calcium channel blocker concentration. CR₂ values were derived by interpolation from these plots and are defined as the concentration of drug required to displace the calcium concentration-response curve two fold. By use of this protocol, the potencies of verapamil, nifedipine and diltiazem were determined in both

‘normal’ and ‘ischaemic’ (metabolic acidosis, hypoxia and 9.2 mM K⁺) conditions. Finally the CR₂ value for verapamil alone was also determined in conditions which comprised respiratory acidosis, hypoxia and 9.2 mM K⁺.

Drugs used

The following compounds were dissolved and diluted in distilled H₂O before use: (±)-verapamil HCl (Sigma), diltiazem HCl (Glaxo Group Research), dinitrophenol (Sigma) and propranolol HCl (ICI). Nifedipine (Sigma) was dissolved in absolute ethanol at a concentration of 10 mM. Further dilutions of nifedipine (100 nM–1 μM) were prepared in distilled H₂O. All drug solutions were prepared freshly, immediately before use. Because of the light-sensitive nature of nifedipine, all experiments were carried out in subdued lighting conditions and the solutions protected from light and kept on ice awaiting use. All chemicals for the physiological salt solutions were of Analar grade and obtained from BDH

Ltd., England. All gas mixtures were obtained from BOC.

Expression of results and statistical analysis

Individual groups of IC_{50} and CR_2 values appeared to be log normally distributed for each group of experiments, as assessed by the Wilks-Shapiro test of normality. Because of this, statistical comparison of sets of individual IC_{50} or CR_2 values was performed by means of parametric statistics (unpaired Student's t test) on the $\log_{10}$ of these values. In addition because the data sets were log normally distributed, mean IC_{50} and CR_2 (Tables 2, 3 and 4) were expressed as geometric means with 95% confidence limits. All other grouped data (e.g. Table 1) were expressed as arithmetic mean values $\pm$ s.e.mean.

Since the Ca^{2+} antagonistic potency of each calcium channel blocking drug under 'normal' and 'ischaemic' conditions was only compared statistically at one point on the concentration-effect relationship (i.e. at the CR_2 level), a fuller statistical analysis which utilised all data points was undertaken. Thus all of the individual CR values for a particular compound, obtained over a series of experiments under 'normal' and 'ischaemic' conditions, were best fitted to two lines by means of a parallel line model. Statistical differences between these two lines were then determined by analysis of co-variance.

In all analyses the level of statistical significance was taken as $P < 0.05$.

Results

The direct negative inotropic effects of the various 'ischaemic' interventions on the force of contraction of guinea-pig electrically stimulated papillary muscles

The various modifications in pH, PO_2 and K^+ caused negative inotropic effects in their own right (Table 1). Combinations of modifications caused decreases in the force of contraction, which were approximately equal to the sum of the decreases in force of contraction caused by the individual interventions alone. Although the negative inotropic effects were relatively large (up to 70% of the starting developed tension in the case of the combination of pH 6.9, 9.2 mM K^+ and hypoxia), after 2.5 h of these conditions (henceforth referred to as 'ischaemia'), preparations which received only vehicle were largely able to recover and maintain (for at least 30 min) their initial developed tension when returned to 'normal' conditions at the end of each experiment

(Table 1). The measured pH values were also very close to those predicted by the Henderson-Hasselbach equation. This suggested that the variously modified physiological salt solutions were fully saturated with the gas mixtures. The hypoxic gas mixture which contained N_2 substituted for some of the O_2 , reduced the normal PO_2 value by about 40% (Table 1).

Verapamil-induced negative inotropic effects

Cumulative addition of verapamil (10 nM–10 μ M) to the papillary muscles caused concentration-related decreases in the developed tension (Figure 1a). Under 'normal' conditions, the mean concentration of verapamil which caused a 50% reduction in developed tension (IC_{50}) was 107 nM ($n = 34$, Table 2). The singular interventions of metabolic acidosis (pH 6.9 or 6.5), elevated K^+ (9.2 mM K^+) or hypoxia

Figure 1 The negative inotropic effects of (a) verapamil, (b) nifedipine, (c) diltiazem and (d) dinitrophenol on guinea-pig electrically stimulated papillary muscles under 'normal' (open symbols) and 'ischaemic' (solid symbols) conditions. Preparations were maintained at 32°C and stimulated at 3 Hz with pulses of 5 ms duration at threshold voltage (approximately 1 V). Concentration-effect curves are the mean from n experiments (a, $n = 10$ –34; b, $n = 5$; c, $n = 6$ –7; d, $n = 7$ –9); vertical lines show s.e.mean.

Table 2 The effect of the various ischaemic interventions upon the negative inotropic potency of verapamil in guinea-pig isolated papillary muscles

Experimental intervention	Verapamil IC ₅₀ (nM)	n
'Normal'	107 (80–142)	34
Mild metabolic acidosis (pH 6.9)	124 (54–295) NS	5
Severe metabolic acidosis (pH 6.5)	106 (40–282) NS	5
9.2 mM K ⁺	134 (36–501) NS	4
Hypoxia	101 (60–169) NS	6
Mild metabolic acidosis + hypoxia	75 (51–111) NS	5
Mild metabolic acidosis + hypoxia + 9.2 mM K ⁺ (‘ischaemia’)	28 (17–48) *	10

IC₅₀ values are geometric mean values (95% confidence interval) from *n* experiments.

Individual log IC₅₀ values for verapamil under each of the various conditions were compared to the log IC₅₀ values of verapamil under the ‘normal’ conditions by unpaired Student’s *t* test.

NS = not significant, * *P* < 0.001.

alone, did not significantly affect this IC₅₀ value (Table 2). In addition, a combination of hypoxia plus metabolic acidosis (pH 6.9), which produced a greater negative inotropic effect than either intervention alone (Table 1), produced a small but not statistically significant decrease in the mean IC₅₀ of

verapamil (Table 2). However a further combination of metabolic acidosis (pH 6.9), elevated K⁺ (9.2 mM) and hypoxia (‘ischaemia’) resulted in a significant reduction in the mean IC₅₀ of verapamil to 28 nM (Table 2; Figure 1a). This effect represented a four fold potentiation of the negative inotropic effect of verapamil.

Effect of stimulation voltage upon the ‘ischaemia’-induced potentiation of the negative inotropic effect of verapamil

Since papillary muscle preparations were stimulated at threshold voltage, it is possible that the ‘ischaemia’ induced a conduction failure which would have been observed as a negative inotropic effect. This ‘ischaemia’-induced negative inotropic effect may have led to a potentiation of the negative inotropic action of verapamil. Experiments were therefore performed with papillary muscles under ‘normal’ and ‘ischaemic’ conditions at both threshold (~1 V) and supra-maximal (5 V) stimulation voltages. However, since at such supra-maximal voltages a significant release of endogenous catecholamine occurs (Blinks, 1966), a further series of studies was performed at both threshold and supra-maximal voltage in the additional presence of propranolol (300 nM). The mean IC₅₀ values from these experiments are shown in Table 3. The negative inotropic potency of verapamil under either ‘normal’ or ‘ischaemic’ conditions varied between the four experimental protocols. For example, there was a significant reduction in the potency of verapamil

Table 3 The effect of raised stimulation voltage in the absence or presence of propranolol upon the negative inotropic potency of verapamil in guinea-pig isolated electrically stimulated papillary muscles under ‘normal’ and ‘ischaemic’ conditions

	‘Normal’ IC ₅₀ (nM)	n	‘Ischaemic’ IC ₅₀ (nM)	n	Ratio N:I
Threshold voltage	107 (80–142)	34	28 (17–48)**	10	3.8 (2.2–6.8)
Threshold voltage + propranolol (300 nM)	65 (33–128)	9	25 (17–37)*	10	2.6 (1.3–5.3)
Supramaximal voltage	338 (150–763)	11	85 (50–146)*	10	4.0 (1.6–10.1)
Supramaximal voltage + propranolol (300 nM)	174 (95–321)	17	41 (29–57)**	9	4.3 (1.8–9.9)

IC₅₀ values are geometric mean (95% confidence interval) from *n* experiments. Individual log₁₀ IC₅₀ values for verapamil under ‘ischaemic’ conditions were compared with the corresponding log₁₀ IC₅₀ values of verapamil under ‘normal’ conditions by means of unpaired Student’s *t* test; ** *P* < 0.001, * *P* < 0.01. Ratio N:I values represent the ratio of the ‘normal’: ‘ischaemic’ mean IC₅₀ values. The 95% confidence interval for this single ratio represents the antilog of the confidence interval derived from unpaired *t* test on the log₁₀ transformed IC₅₀ values under ‘normal’ and ‘ischaemic’ conditions.

Table 4 The effect of 'ischaemia' upon the actions of verapamil, nifedipine, diltiazem and dinitrophenol determined in the guinea-pig isolated papillary muscle

Drug	Direct negative inotropic effect			Antagonism of positive inotropic effect of Ca^{2+}					
	'Normal'	n	IC_{50}^a (nM) 'Ischaemia'	Ratio N:I	'Normal'	n	CR_2^b (nM) 'Ischaemia'	n	Ratio N:I
Verapamil	107 (80-142)	34	28* (17-48)	3.8 (2.2-6.8)	467 (237-920)	5	50** (17-147) 50*** (16-156)	4 4 4	9.3 (3.2-27.5) 9.3 (3.1-28.2)
Nifedipine	28 (22-35)	5	24 ^{NS} (15-36)	1.2 (0.8-1.8)	34 (16-72)	5	29 ^{NS} (9-95)	4	1.2 (0.4-3.9)
Diltiazem	1380 (516-3700)	6	570 ^{NS} (270-1200)	2.4 (0.8-7.2)	2454 (162-37,200)	4	1080 ^{NS} (448-2581)	4	2.3 (0.1-39.8)
Dinitrophenol ^c	2560 (1020-6450)	7	4760 ^{NS} (2770-8200)	0.5 (0.2-1.4)	NT	NT	NT	—	—

IC_{50} and CR_2 values are geometric means (95% confidence interval) from n experiments. Ratios N:I values represents the ratio of the 'normal': 'ischaemic' mean IC_{50} or CR_2 values. The 95% confidence interval for this single ratio represents the antilog of the confidence interval derived from unpaired t test on the $\log_{10}$ transformed IC_{50} or CR_2 values under 'normal' and 'ischaemic' conditions for each drug.

* Concentration that reduced the developed tension of papillary muscle by 50%.

^b Concentration that produced a 2 fold rightward displacement of a Ca^{2+} positive inotropic concentration-effect curve.

^c IC_{40} values shown since highest concentration of dinitrophenol used did not always produce 50% reduction in developed tension.

^d Ischaemic conditions comprised of respiratory acidosis with hypoxia and elevated $[K^+]$ instead of metabolic acidosis, hypoxia and elevated $[K^+]$. Individual $\log IC_{50}$ or CR_2 values for each drug under 'ischaemic' conditions were compared with the corresponding $\log$ values obtained under 'normal' conditions by means of unpaired Student's t test.

NS = not significantly different; * $P < 0.001$; ** $P < 0.0001$.

NT = not tested.

Figure 2 The effects of Ca^{2+} on developed tension of guinea-pig electrically stimulated papillary muscles. Ca^{2+} concentration-effect curves were constructed under either 'normal' (∇) or 'ischaemic' ($\blacktriangledown$) conditions. For stimulation parameters see Figure 1. Concentration-effect curves are the mean of 6 experiments in each case; vertical lines show s.e.mean.

Figure 3 The effect of verapamil upon the positive inotropic effect of Ca^{2+} in guinea-pig isolated electrically stimulated papillary muscle. Curves shown represent the mean ($n = 4-5$) third Ca^{2+} concentration-effect curve performed on separate preparations. Responses were expressed as a percentage of the 9 mM Ca^{2+} response in the second curve (see Methods). Curves shown are those performed in the presence of vehicle ($\square$) or (a) verapamil (100 nM, $\blacksquare$; 1000 nM, $\bullet$ or 3000 nM, $\blacktriangle$) under 'normal' conditions or (b) verapamil (10 nM, $\triangle$; 100 nM, $\blacksquare$ or 1000 nM, $\bullet$) under 'ischaemic' conditions. Vertical lines show s.e.mean.

($P < 0.01$) at 5 V compared to threshold voltage of stimulation under either 'normal' (338 vs 107 nM) or 'ischaemic' (85 vs 28 nM) conditions. A small but significant ($P < 0.05$) reduction in potency was also observed under 'normal' or 'ischaemic' conditions at 5 V compared with threshold voltages in the presence of propranolol. However, in all instances, the IC_{50} values under 'ischaemic' conditions were significantly ($P < 0.01$) less than the corresponding values under 'normal' conditions (Table 3). This potentiation of the negative inotropic effect of verapamil by 'ischaemia' was very reproducible between the various protocols (Table 3).

Nifedipine, diltiazem and dinitrophenol

Nifedipine (10 nM–10 μM) and diltiazem (0.1–10 μM) caused concentration-related negative inotropic effects in both 'normal' and 'ischaemic' conditions (Figure 1b and c). Unlike verapamil, the negative inotropic effect of nifedipine was unaltered by the 'ischaemic' conditions (Figure 1b, Table 4). Although the negative inotropic effect of diltiazem measured at the IC_{50} level was potentiated some 2–3 fold by the 'ischaemic' conditions this change did not achieve statistical significance ($P = 0.09$) (Figure 1c, Table 4).

The finding that the negative inotropic effect of the calcium channel blocking drugs was affected differentially was unexpected. However, since the combination of interventions constituting 'ischaemia' produced such a profound negative inotropic effect itself, it was conceivable that this effect itself was in some way responsible for the potentiation observed. As a test of specificity the negative inotropic effects of a non-calcium channel blocking drug, dinitrophenol, were examined under 'normal' and 'ischaemic' conditions. Over the concentration range 0.1–10 μM , this agent produced negative inotropic effects with an IC_{50} under 'normal' conditions of 2.6 μM . Interestingly, unlike verapamil, the negative inotropic effect of this compound was not potentiated by the 'ischaemic' conditions compared to 'normal' conditions (Figure 1d, Table 4).

Drug-induced antagonism of the positive inotropic effect of Ca^{2+} in 'normal' and 'ischaemic' conditions

Determination of direct negative inotropic potency was difficult because the residual developed tension after application of the 'ischaemic' conditions was relatively small. As an alternative procedure, the antagonistic effect of the three calcium channel blockers upon the positive inotropic effect of exogenously added Ca^{2+} was therefore investigated. Whilst Ca^{2+} (1.0–9 mM) caused a concentration-

related increase in developed tension in both 'normal' and 'ischaemic' conditions, which was highly reproducible in successive curves, the overall change in developed tension was smaller in 'ischaemic' than in 'normal' conditions for any given concentration of Ca^{2+} (Figure 2). Verapamil (0.1–3 μM) produced concentration-related rightward displacements of the Ca^{2+} concentration-response curve in 'normal' conditions (Figure 3a). However, the antagonistic activity of verapamil was greater under 'ischaemic' conditions (Figure 3b). This increase in potency is more clearly seen in Figure 4a which is a plot of verapamil concentration versus its effect (Ca^{2+} CR values) derived from the individual experiments shown in Figure 3a and b. From this plot the CR_2 (concentration to produce a two fold rightward displacement of the Ca^{2+} concentration-effect curve) for verapamil was found to be approximately 9 fold less under 'ischaemic' compared with 'normal' conditions (Table 4).

As an alternative to the metabolic acidosis used in the 'ischaemic' conditions, in a further series of experiments, the pH of the physiological salt solution was reduced by bubbling the solution with a gas mixture containing 17.5% CO_2 , 55% O_2 and 27.5% N_2 . Effects produced by these conditions are shown in Table 1 (H). Under these 'ischaemic' conditions with the lowered pH component represented by this respiratory acidosis, the mean CR_2 for verapamil was reduced to 50 nM (Table 4). This value was not significantly different from the CR_2 obtained for verapamil under the 'ischaemic' conditions incorporating metabolic acidosis and represented an approximate 9 fold potentiation compared with 'normal' conditions (Table 4).

Diltiazem (2–20 μM) and nifedipine (0.03–1 μM) also produced concentration-related rightward displacements of the Ca^{2+} concentration-response curves and the concentration-response relationships for both drugs under 'normal' and 'ischaemic' conditions are shown in Figure 4b and c. Under 'ischaemic' conditions the CR_2 value for diltiazem was decreased 2–3 fold whilst that of nifedipine was unaltered. Since statistical analysis of these data (Table 4) only considered a single point (i.e. the CR_2 value) on the concentration-effect curve for each drug, the whole of the CR data under 'normal' and 'ischaemic' conditions were analysed by means of an analysis of co-variance. This analysis confirmed the highly significant potentiation of verapamil ($P < 0.001$) and the lack of potentiation of nifedipine. However, by use of this more comprehensive analysis a significant potentiation ($P < 0.01$) of diltiazem was revealed.

Table 4 summarises the potencies of the test compounds in both 'normal' and 'ischaemic' conditions obtained by both methods of measuring Ca^{2+} antagonistic potency. A good correlation was

Figure 4 The concentration-effect relationship of (a) verapamil, (b) diltiazem, and (c) nifedipine, for antagonism of the positive inotropic effect of Ca^{2+} in guinea-pig isolated, electrically stimulated papillary muscle under 'normal' (open symbols) and 'ischaemic' (solid symbols) conditions. Data, which were taken from experiments such as those depicted in Figure 3, are expressed as $\log_{10}$ Ca^{2+} CRs versus concentration of antagonist (for calculation of CRs see Methods). Stimulation parameters as in Figure 1. Each point is the arithmetic mean of the $\log_{10}$ of each CR value from 4–6 experiments. These data were best fitted by a parallel line model. Statistical differences between these lines relating to data obtained under 'normal' and 'ischaemic' conditions for each drug were determined by analysis of co-variance. Statistical differences between drug potency in 'normal' and 'ischaemic' conditions were also assessed at the CR_2 level (see legend to Table 4). Vertical lines show s.e.mean.

observed between the two protocols, both in the rank order of calcium channel blocker potency in 'normal' conditions (nifedipine > verapamil > diltiazem) and for the differential sensitisation of drug potency induced by 'ischaemia' (verapamil > diltiazem > nifedipine $\approx$ 0).

Discussion

In the present study, the negative inotropic activities of the calcium channel blocking drugs, verapamil, diltiazem and nifedipine were quantified in guinea-pig isolated, electrically driven papillary muscles by two methods. Regardless of the method used, either direct negative inotropic activity or antagonism of the positive inotropic effect of exogenous Ca^{2+} , the rank order of potency was nifedipine > verapamil > diltiazem. Under various experimental conditions, namely hypoxia, acidosis and elevated extracellular K^+ , designed to mimic the consequences of ischaemia *in vivo*, the negative inotropic potency and Ca^{2+} antagonistic activity of verapamil were found to be significantly potentiated. However, this potentiation was observed only when a combination of these conditions was applied, not when they were applied singly. In addition, under this combination of conditions the activity of the three calcium channel blockers studied were differentially affected. In short, whilst the potencies of verapamil and diltiazem increased, that of nifedipine was unaltered.

Several previous *in vivo* studies have examined the effect of regional myocardial ischaemia upon the negative inotropic potency of calcium channel blocking drugs. Some studies have shown a potentiating effect of ischaemia on drug potency (Smith *et al.*, 1976; Abrahamsson *et al.*, 1985). However, others have failed to demonstrate such an effect (Sherman *et al.*, 1981; Romson *et al.*, 1986). There have also been various studies, which have utilised isolated perfused cardiac muscle, that have confirmed a potentiating effect of ischaemia upon calcium channel blocking potency (Ngai *et al.*, 1983; Konishi *et al.*, 1984). However, none of these studies has attempted to examine systematically the contributory factors occurring during ischaemia which lead to this potentiating effect. In the cat isolated papillary muscle, Briscoe & Smith (Briscoe & Smith, 1982; Smith & Briscoe, 1985) examined the effect of changes in the pH of the bathing medium from 7.4 to 6.0 upon the negative inotropic potency of a range of calcium channel blocking drugs. More recently, Curtis & Walker (1986; 1988) also studied the effect of increasing buffer K^+ on the negative inotropic

potency of verapamil and nifedipine in rat isolated Langendorff perfused ventricles. However, in the ischaemic myocardium *in vivo*, changes in local pH and elevation in extracellular K^+ do not occur singly but do so in tandem. Therefore, in the present study, we attempted to mimic more comprehensively the resultant biochemical sequelae of ischaemia, and thus considered, the combined effects of acidosis elevated extracellular K^+ and reduced O_2 availability on the negative inotropic potency of the calcium channel blockers *in vitro*.

The level of hypoxia chosen was largely dictated by the negative inotropic effect produced by the reduction of Po_2 in the bathing solution. Under normal conditions the Po_2 was 500–600 mmHg, whereas during the conditions termed 'hypoxic' the value was reduced to approximately 350 mmHg. This solution is therefore not truly 'hypoxic', since the Po_2 of arterial blood is only 95 mmHg. However, since the papillary muscle preparation receives oxygen only by diffusion this reduction in Po_2 was enough to compromise functional performance. The choice of the type of acidosis also required some consideration, since there is some controversy as to whether myocardial ischaemia is associated with a metabolic or respiratory acidosis (Ichihara *et al.*, 1984; Kodama *et al.*, 1984). Both types of acidosis were therefore investigated upon the Ca^{2+} antagonist activity of verapamil. The level of acidosis and K^+ concentration chosen for the current experiments, 6.9–7.0 and 9.2 mM respectively, are close to levels measured in the myocardium *in vivo* shortly after total coronary occlusion (Weiss *et al.*, 1984; Hill & Gettes, 1980). We were, however, limited in the levels of acidosis and K^+ concentration that we could choose by the magnitude of the negative inotropic effects produced by the interventions themselves. This was especially the case when we were attempting to quantify the potencies of the calcium channel blocking drugs by measurement of their negative inotropic effects. It was also interesting to note that although the 'ischaemic' conditions used caused a sustained 70% decrease in the developed tension of the guinea-pig papillary muscle, those preparations which received only vehicle and were returned to 'normal' conditions at the end of the experiment (between 2–3 h later) generally regained and maintained their original developed tension (Table 1). This suggests that the 'ischaemic' conditions imposed were not severe enough to cause irreversible functional damage to the muscle.

In the present study it was surprising to find that the negative inotropic effect of verapamil was not significantly potentiated by either mild (pH 6.9) or severe (pH 6.5) acidosis alone or by elevated extracellular K^+ . These results contrast with the findings of Smith & Briscoe (1985) and Curtis & Walker

(1986). In the case of the former study it may be significant that the pH of the bathing medium was reduced to as low as 6.0. In the case of the study of Curtis & Walker (1986), these authors showed that the greatest potentiation of the negative inotropic effect of verapamil occurred at K^+ concentrations up to 5.9 mM, the starting concentration in the present study. Above this concentration the degree of potentiation of verapamil decreased. This, along with the fact that these authors utilised a perfused cardiac muscle preparation from the rat, paced at a higher frequency than used in the present work, may explain the discrepancy between the two studies.

A further combination of hypoxia and 'mild' acidosis still failed to affect significantly the potency of verapamil although a slight trend was observed. In fact, it was only when a combination of mild acidosis, elevated K^+ and hypoxia was used that a significant potentiation of the negative inotropic effects of verapamil was observed. In addition, the nature of the acidosis used in this combination did not appear to be critical. Thus, both mild (pH 6.9–7.0), metabolic or respiratory acidosis potentiated equally the verapamil-induced antagonism of the positive inotropic effect of Ca^{2+} . Interestingly, the critical combination of conditions necessary to observe potentiation of verapamil's effects in the present study has also been found to be necessary to achieve electrophysiological changes in cardiac muscle *in vitro* similar to those seen in ischaemic muscle *in vivo*. Thus, in guinea-pig papillary muscle (Kodama *et al.*, 1984) or in porcine heart (Moréna *et al.*, 1980), a combination of elevated extracellular K^+ , hypoxia and acidosis produced a much more pronounced effect upon the action potential than did the individual interventions themselves. The effects observed with the combination of interventions, namely a reduced maximum upstroke velocity and shortened action potential duration, also mirrored more precisely the type of cardiac action potential measured during coronary artery occlusion (Cinca *et al.*, 1980). Both this observation and those of the present study therefore emphasise the importance of considering all of the integral components present during ischaemia in cardiac muscle when attempting to mimic the situation *in vitro*.

A further unexpected finding of the present study, was the lack of potentiation of the Ca^{2+} antagonistic actions of nifedipine. A rank order of potentiation of verapamil > diltiazem > nifedipine also contrasts with that obtained by Smith & Briscoe (1985). Whilst differences in species and experimental conditions may have contributed to this inconsistency, it is interesting to note other findings of an identical order of potentiation of these three compounds to that obtained in the present study. In each of these studies, which concerned either anoxia-induced

damage of rat myocytes (Miller *et al.*, 1985), necrotic changes in the myocardium of cardiomyopathic hamsters (Jasmin & Proschek, 1984) or veratrine-induced Ca^{2+} overload in rat myocytes (Ver Donck *et al.*, 1986), verapamil was more protective than diltiazem, whilst nifedipine (or a similar dihydropyridine, nisoldipine) afforded no protection at all. The similarity of these results with the rank order of 'ischaemia'-induced increase in potency of the same compounds in the present study may be coincidental, but is striking nevertheless.

The present study raises two obvious questions. Firstly, what is the mechanism by which the potency of verapamil is increased in the 'ischaemic' conditions and secondly, why is the potency of nifedipine not changed? Initially, upon observing the 'ischaemia'-induced potentiation of the negative inotropic effect of verapamil, we wondered whether conduction failure was occurring in the papillary muscles under the 'ischaemic' conditions and that it was this that was somehow producing the potentiation observed. However, in a series of experiments with increased stimulation strength to overcome any conduction failure, the 'ischaemia'-induced potentiation was consistently observed, so ruling out the involvement of such a mechanism. In these experiments, with increasing stimulation voltage a small but significant reduction was always observed in the negative inotropic potency of verapamil, when compared with the value obtained under the corresponding experimental conditions at threshold voltage. This could imply that at threshold voltage, under either 'normal' or 'ischaemic' conditions, part of the negative inotropic effect of verapamil was exerted through a negative dromotropic action. Under conditions of supramaximal stimulation such an effect would be overcome and hence the negative inotropic potency of verapamil reduced. However, from the results obtained at higher stimulation voltages this negative dromotropic action of verapamil would also appear to be unrelated to the 'ischaemia'-induced potentiation observed.

Further confirmatory evidence that conduction failure was not responsible for the potentiating effect of 'ischaemia' was the finding that neither nifedipine nor dinitrophenol were affected under these conditions. This observation would also seem to rule out the possibility that the negative inotropic effect induced by the 'ischaemic' interventions themselves, somehow facilitated the negative inotropic action of verapamil, since other negative inotropic agents would also be expected to be potentiated by such a mechanism. Thus, an alternative explanation must be sought for the differential potentiation of the calcium channel blocking drugs seen in the present study.

Calcium channel blocking drugs can be classified

according to their properties as either voltage-dependent or use (frequency)-dependent. The three compounds tested in the present study are all voltage-dependent (Kanaya *et al.*, 1983; see Chin, 1986): that is, as the membrane potential become less negative, calcium channel blocking potency is enhanced. If 'ischaemia' led to a partial membrane depolarisation in the cardiac cells, a generalised potentiation of the calcium channel blocking activity of all of the compounds would have been anticipated. However, this was not the case. In contrast, the so-called use- or frequency-dependency is more a property of verapamil than of nifedipine. One interpretation of this use/frequency-dependency is that a compound which has its binding site either within the cell or in the calcium channel itself requires channel opening to gain access to this site (Katz, 1985; Frank, 1986). There is now good evidence that the verapamil binding site associated with the calcium channel in the mammalian myocardium is on the inner surface of the cell membrane (Heschler *et al.*, 1982). As mentioned, dihydropyridines such as nifedipine, exhibit less use-dependency than verapamil (Lee & Tsien, 1983 see Curtis & Walker, 1988). One explanation for this is that, as neutral entities, they are highly lipid soluble and so should easily gain access to their binding site independent of

channel opening (Kokubun & Reuter, 1984). The 'ischaemia'-induced potentiation of verapamil observed in the present study could suggest that more verapamil gains access to its binding site, possibly as a consequence of a perturbation of the cell membrane. Such a change would not be anticipated to affect the potency of nifedipine.

In conclusion, in an *in vitro* model of ischaemia, the present study has demonstrated a potentiation of the calcium channel blocking activity of verapamil thus, confirming previous findings *in vivo*. In contrast to other *in vitro* studies, the phenomenon appears to depend upon a critical combination of conditions and, interestingly, does not apply universally to all calcium channel blocking drugs. Elucidation of the mechanisms involved may yield important information regarding the future design of compounds with marked selectivity of action in the ischaemic over the normally perfused myocardium. Such selectivity may be of benefit in reducing myocardial infarct size or reducing the incidence of the arrhythmias of early myocardial ischaemia (Curtis & Walker, 1988).

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