British Journal of Pharmacology (1997) 120, 19–24

Calcium-antagonist effects of norbormide on isolated perfused heart and cardiac myocytes of guinea-pig: a comparison with verapamil

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1 Cardiac effects of norbormide and verapamil were compared in single ventricular myocytes, right atria, and Langendorff perfused hearts isolated from guinea-pigs.

2 In ventricular myocytes, norbormide 50 μM inhibited the peak calcium current (I_{Ca}) by 49.6±3.9% without altering the shape of the current-voltage relationship; verapamil 1 μM inhibited I_{Ca} by 83.2±3.3%. Neither norbormide nor verapamil affected I_{Ca} at the first beat after a 3 min quiescence period; during repeated depolarizations, both drugs cumulatively blocked I_{Ca} (use-dependence), with time constants of 23.0±7.0 s for norbormide and 91.3±8.4 s for verapamil.

3 In constant-flow perfused hearts electrically driven at 2.5 Hz or 3.3 Hz, both norbormide and verapamil concentration-dependently decreased ventricular contractility (dP/dt_{max}), atrio-ventricular (AV) conduction velocity and coronary pressure. Intraventricular conduction velocity was slightly decreased by norbormide but not by verapamil. At an equivalent change in AV conduction, norbormide depressed heart contractility less than verapamil. The effects of norbormide on AV conduction, intraventricular conduction, and contractility were frequency-dependent. Furthermore, the curves correlating the mechanical and electrical effects of norbormide at the two frequencies used were apparently coincident, while those of verapamil were clearly separated.

4 In spontaneously beating right atria, norbormide and verapamil decreased the frequency of sinus node (SA) in a concentration-dependent way. At an equivalent effect on the AV conduction, norbormide exerted a greater effect on sinus frequency than verapamil.

5 These results indicate that in guinea-pig heart norbormide has the pharmacological profile of a Ca-antagonist with strong electrophysiological properties. In comparison with verapamil, norbormide is more selective on SA and AV node tissues and exerts a weaker negative inotropic effect on ventricles. In principle, this pattern of effects may be an advantage in treating supraventricular tachyarrhythmias in patients with heart failure. The effect of norbormide on intraventricular conduction may represent an additional antiarrhythmic mechanism.

Keywords: Norbormide; verapamil; calcium-antagonists; isolated perfused heart; L-type calcium current

Introduction

Norbormide (Figure 1) is a unique vasoactive compound inasmuch as it can exert either vasoconstrictor or vasorelaxant effects depending on the animal species and the calibre of the vessel. In rats norbormide selectively contracts small calibre vessels (Roszkowski *et al*., 1964; Roszkowski, 1965; Bova *et al*., 1996) and relaxes large size vessels (e.g. thoracic aorta) contracted with high-K^+ (Bova *et al*., 1996). In vascular preparations from other species (guinea-pig, mouse, man), norbormide has no effect on resting tone and causes relaxation of high-K^+ contracted vessels (Bova *et al*., 1996). The mechanisms of these opposite effects have been investigated recently and there is evidence that the vasoconstriction is mediated by calcium influx through plasmalemmal calcium channels and vasorelaxation is due to a blockade of the voltage-dependent calcium channel (Bova *et al*., 1996). This latter property may have, in principle, therapeutic applications and prompted us to investigate whether norbormide also exerts a Ca-antagonistic action at the cardiac level. Hence, we studied the effect of norbormide on the mechanical and electrophysiological activities of guinea-pig myocardium, comparing the pattern of the effects of norbormide with those of verapamil, taken as a reference calcium-antagonist.

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Methods

Voltage clamp experiments

Cell isolation Calcium tolerant ventricular myocytes were isolated by a modification of the procedure described by De Young *et al*. (1989). Briefly, hearts of male guinea-pigs (250–300 g), treated with heparin (5000 iu kg^{-1}) and anaesthetized with zolazepanum 8 mg kg^{-1} + tiletaminum 12 mg kg^{-1}, were removed and retrogradely perfused (8 ml min^{-1}) with Ca^{2+}-free Joklik medium (Sigma Chemie GmbH) supplemented with 10 mM taurine. The perfusate was then changed to a Joklik medium containing 0.4 mg ml^{-1} type A collagenase, 0.2 mg ml^{-1} trypsin, 1 mg ml^{-1} BSA fraction V (Boehringer Mannheim GmbH, Germany) and recirculated for 20 min. Afterwards, $CaCl_2$ concentration was increased in steps to 0.25, 0.5, 1 and 1.25 mM at 5 min intervals. The ventricles were then cut into small pieces, placed into fresh enzyme mixture and incubated at 37°C in a shaker bath until the ventricular fragments were totally digested (10–15 min). Supernatant cell suspension was washed, resuspended in Ca^{2+}-Joklik medium and layered on Percoll (Pharmacia Fine Chemicals,

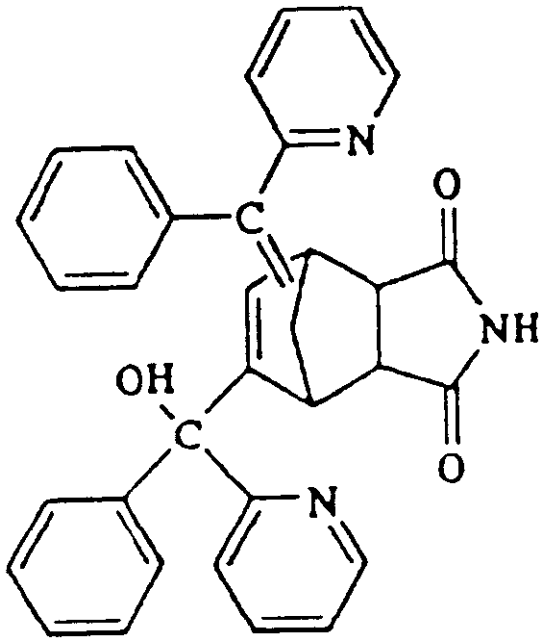

Figure 1 Structure of norbormide.

Upsala, Sweden). The cells were recovered from the pellet, washed and resuspended in the culture medium.

Electrophysiological experiments

The whole cell recording method was used to measure calcium current (I_{Ca}). The composition of the patch pipette solution was (mM): KCl 133, EGTA 5, Na_2ATP 5, Na_2GTP 0.4, phosphocreatine-Na_2 5, $MgCl_2$ 3 and HEPES 5 (pH 7.2). The cells were superfused with a solution containing (mM): Nacl 137, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 5.5 and HEPES 10 (pH 7.4). Because we wanted to study the effects of norbormide in a relatively physiological state, we did not remove sodium or replace potassium with caesium in bath or pipette. The experiments were performed at room temperature (22–23°C). Voltage clamp protocols were applied to the myocytes by means of a patch clamp amplifier (Axopatch-1C, Axon Instruments). L-type calcium current was elicited by depolarizing pulses of 280 ms duration applied with a frequency of 0.2 Hz from a holding potential of −40 mV up to +10 mV. The −40 mV holding potential excluded the T-type Ca current and the inward Na current. The current-voltage relations were obtained with voltage pulses up to +60 mV in 10 mV increments. To evaluate the relative contribution of the tonic and the use-dependent component of calcium channel blockade induced by norbormide and verapamil, I_{Ca} was also measured during repetitive depolarizations (0.05 Hz) after a 3 min quiescent period.

Langendorff isolated perfused hearts

Guinea pigs of either sex (300–400 g) were killed by cervical dislocation. The hearts were quickly removed and rapidly perfused through the aorta at constant flow (8–9 ml g^{-1} tissue - min^{-1}) with a modified Krebs-Henseleit solution of the following composition (mM):NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaCO_3$ 25, KH_2PO_4 1.2, glucose 11.1 and Na pyruvate 2, bubbled with an O_2:CO_2 gas mixture (95%-5%) (pH 7.4 ± 0.01) and kept at 37°C. After a stabilization period of 30 min, the sinus node was excised and the hearts were driven at the frequency of 2.5 Hz, through platinum electrodes placed on the left atrium. Heart contractility was measured, by means of an intraventricular rubber balloon, as the maximal positive derivative of the left ventricle pressure ($+dP/dt_{max}$). Surface ECG was recorded by means of two electrodes, one placed on the crux cordis and the other on the left ventricle free wall (Yang *et al*., 1995).

The signal was amplified and monitored with a digital memory oscilloscope (PM 3331, Philips), connected to a printer. The main ECG intervals (PR = atrio-ventricular conduction time; QRS = intra-ventricular conduction time; JT = duration of ventricular depolarization) were measured directly on the screen by use of vertical cursors. The drug-induced changes in conduction velocity of AV node and ventricular myocardium were calculated as changes in the reciprocal of the PR interval and QRS interval, respectively. Coronary perfusion pressure (cm H_2O) was monitored to assess the change in coronary vessel resistance.

Hearts were stepwise exposed to the following norbormide concentrations: 2.5, 5, 10, 25 and 50 μM. At each drug concentration, the effects were measured after an equilibration time of 10 min. In a separate set of experiments the cardiac effects of the reference calcium-antagonist verapamil were also evaluated at concentrations of 0.05, 0.1, 0.25, 0.5 and 1 μM (equilibration time: 15 min). To evaluate the dependence of drug effects on stimulation frequency, after the effect of each drug concentration was stabilized, heart rate was increased from 2.5 Hz to 3.3 Hz until a new pharmacodynamic equilibrium was reached (usually within 2 min from rate change). Before the next drug addition, frequency was again set at 2.5 Hz. The limited range of frequencies employed was dictated by the electrophysiological characteristics of the guinea-pig isolated heart: at frequencies lower than 2.5 Hz junctional escape beats often occur and at frequencies higher than 3.3 Hz the T wave end usually merges with the following P wave, making it difficult to measure PR and JT intervals.

Isolated right atrium preparations

Guinea-pig hearts were removed as described above and put in an ice-cold physiological solution. Right atrium was excised and vertically suspended in a 10 ml Blink bath at 35°C and connected to a isometric force transducer coupled to a pen recorder. The physiological salt solution had the same composition as described for the isolated heart. The effects of norbormide and verapamil on spontaneous sinus rate were measured at the same concentrations tested in the Langendorff preparations.

Statistical analysis

The values of the parameters measured were presented as mean ± s.e. Comparisons between paired data were carried out by means of Student's paired *t* test. The concentration-effect curves obtained at different driving frequencies were compared by the ANOVA. *P* values <0.05 were considered statistically significant.

Drugs

Norbormide was a gift from I.N.D.I.A. Industrie Chimiche (Padova, Italy); verapamil hydrochloride was purchased from Sigma Chimica (Milano, Italy). Stock solutions of norbormide (50 mM) and verapamil (10 mM) were prepared in dimethylformamide and distilled water, respectively. At the maximal concentration (1 μl ml^{-1}) reached in the medium, dimethylformamide had no effect on the parameters measured.

Results

I_{Ca} *measurement*

In guinea-pig ventricular myocytes 50 μM norbormide inhibited the peak I_{Ca} by 49.6 ± 3.9% ($P<0.001$, $n=7$) without influencing its decay (relaxation $t_{½}$: 13.4 ± 3.1 ms vs 11.01.6 ms, NS) (Figure 2a). Maximal inhibition occurred after drug exposure of 5–7 min and, after 8 min washout, calcium current recovered to 65.6 ± 5.1% of its control value.

The current-voltage relationship of I_{Ca} was uniformly depressed by norbormide and the potential at which I_{Ca} was maximal did not change (Figure 2b). Verapamil (1 μM) inhibited the peak calcium current by 83.2 ± 3.3% and, similar to norbormide, did not alter the shape of the current-voltage relationship (data not shown). The experiments carried out to evaluate the use-dependence of I_{Ca} block showed that both norbormide and verapamil minimally affected I_{Ca} at the first beat applied after the quiescent period (Figure 3a and b). Ca current blockade cumulatively developed during repeated depolarization (use-dependence) and the time constant of the process was considerably shorter for norbormide (23.0 ± 7.0 s) than for verapamil (91.3 ± 8.4 s).

Langendorff isolated perfused hearts

The mean (± s.e.) baseline values of the cardiac parameters measured at 2.5 Hz and 3.3 Hz (dP/dt_{max}, coronary pressure, PR, QRS, JT intervals) are shown in Table 1. Since control parameters did not differ significantly between verapamil and norbormide groups, they were pooled. Both verapamil and norbormide decreased dP/dt_{max} (Figure 4a), coronary pressure (Figure 4b) and AV conduction velocity (Figure 5a) in a concentration-dependent way. Intraventricular conduction velocity was slightly decreased by only norbormide (Figure 5b) and neither drug modified JT interval (data not shown). The effects of either drug on AV conduction were frequency-dependent (Figure 5a) and in the presence of the highest verapamil concentration (1 μM) a complete AV block ensued in 3 out of 5 experiments when the stimulation frequency was increased from 2.5 Hz to 3.3 Hz. For this reason the corresponding data concerning coronary and contractility effects were omitted in Figure 4. No significant differences in the coronary effects of the two drugs were detected between 2.5 Hz and 3.3 Hz (Figure 4b). A significantly greater effect on contractility at the highest frequency was observed only with norbormide (Figure 4a). Independent of heart rate, contractility was completely depressed by 1 μM verapamil, while norbormide, at the maximal

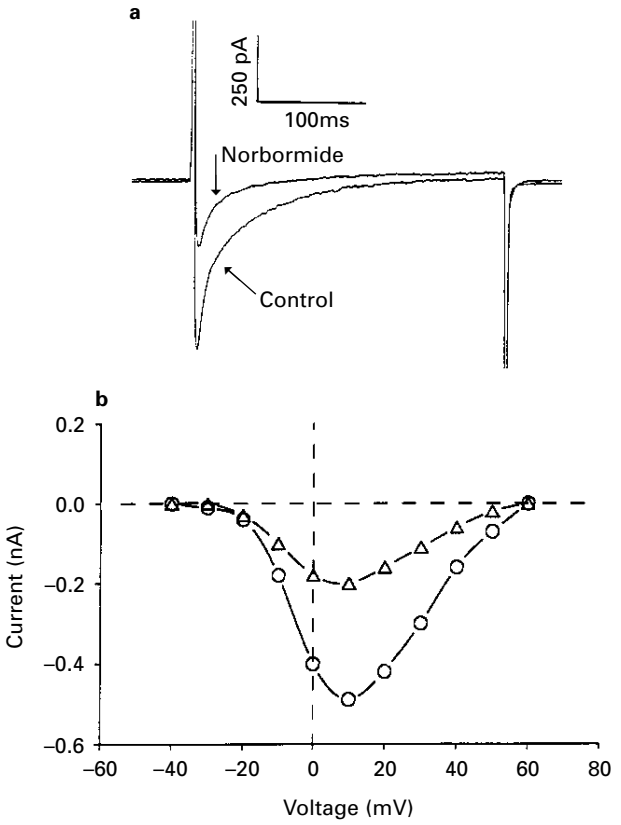

Figure 2 (a) Tracings showing the effect of 50 μM norbormide on I_{Ca} elicited in ventricular myocytes by 280 ms pulses to −10 mV from a holding potential of −40 mV. (b) Steady-state current-voltage relationship of I_{Ca} in control conditions (○) and in the presence of 50 μM norbormide (△). The test pulses were applied from a holding potential of −40 mV to various potentials, with 10 mV steps.

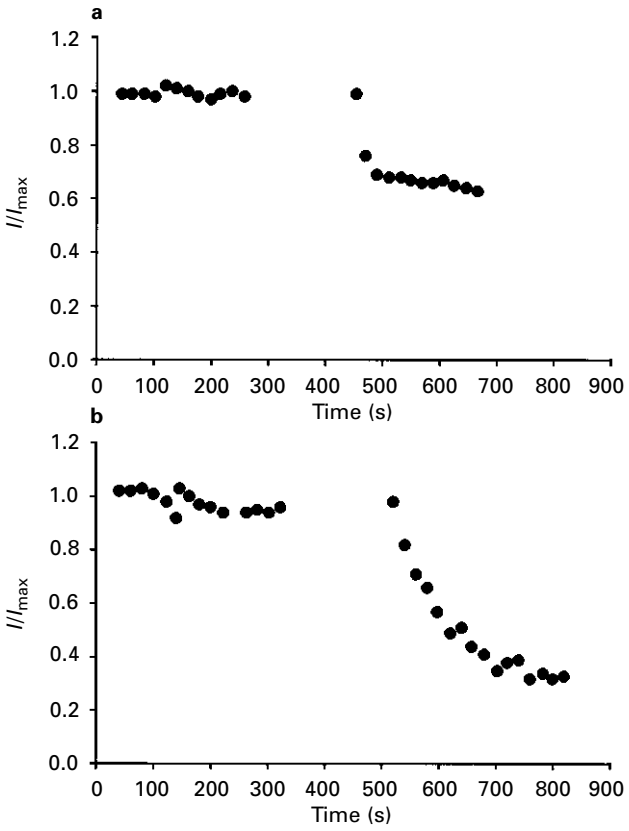

Figure 3 Effect of repetitive depolarizations (280 ms test pulses from −40 to +10 mV, applied every 20 s) on the development of I_{Ca} blockade by 50 μM norbormide (a) and 1 μM verapamil (b). Current changes are expressed as a fraction of the maximal I_{Ca} recorded in control conditions (I/I_{max}). I_{Ca} was measured before adding the drug and after a 3 min drug exposure during which the cell was not depolarized. In these experiments the time constants of the block development are 17 s and 88 s for norbormide and verapamil, respectively.

Table 1 Baseline values of the parameters measured in isolated perfused hearts driven at 2.5 and 3.3 Hz

Driving frequency (Hz)	dP/dt_{max} ($mmHg\,s^{-1}$)	*Coronary pressure* ($cm\,H_2O$)	*PR* (ms)	*QRS* (ms)	*JT* (ms)
2.5	1351 ± 105	65.5 ± 7.5	44.0 ± 2.3	18.5 ± 2.1	157.5 ± 6.3
3.3	1516 ± 130	63.5 ± 7.9	48.1 ± 2.0	18.5 ± 2.1	133.9 ± 5.4

Data shown are means ± s.e.mean, $n = 5$.

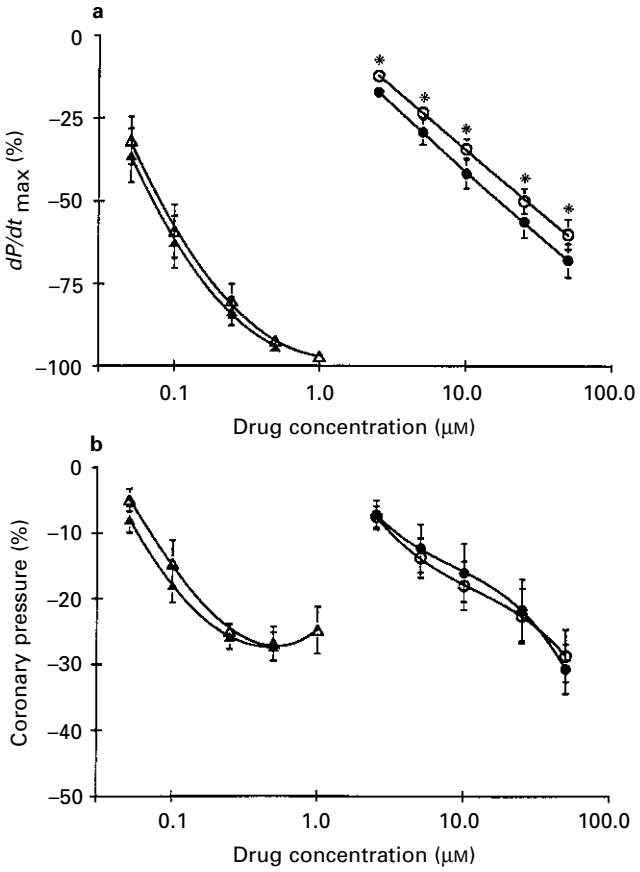

Figure 4 Concentration response curves for verapamil (△, ▲) ($n=5$) and norbormide (○, ●) ($n=5$) for their negative inotropic effects (a) and coronary vasodilator effects (b). The effects are expressed as percentage changes from control values at two stimulation frequencies: 2.5 Hz (open symbols) and 3.3 Hz (solid symbols). Asterisks indicate the significance ($P \leqslant 0.05$) of the frequency-dependent changes. Error bars that fall within the symbols are not shown.

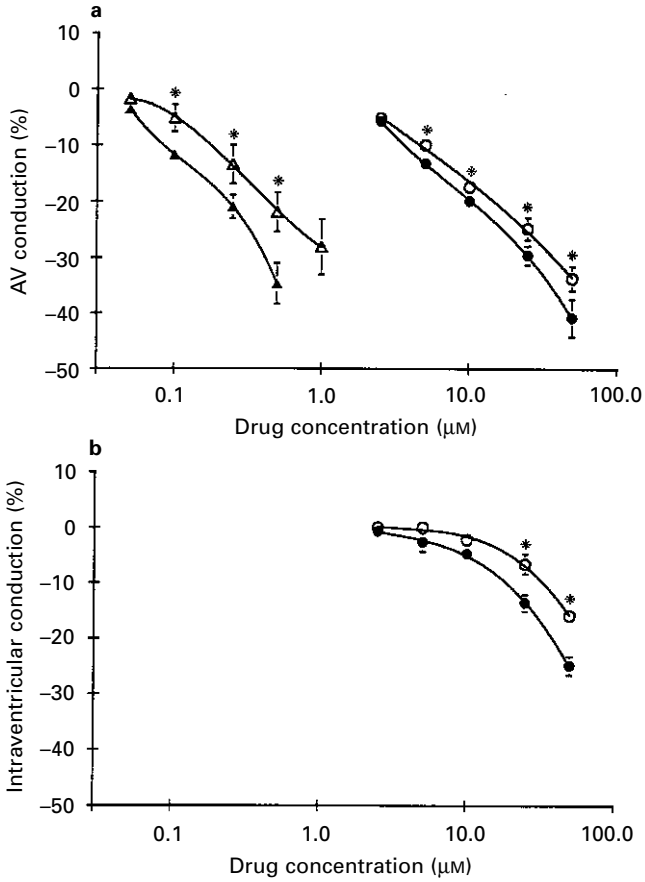

Figure 5 (a) Concentration response curves for verapamil (△, ▲) ($n=5$) and norbormide (○, ●) ($n=5$) for their negative dromotropic effect on the AV node. (b) effect of norbormide on the intraventricular conduction velocity (not affected by verapamil). The effects are expressed as percentage changes from the control values at two stimulation frequencies: 2.5 Hz (open symbols) and 3.3 Hz (solid symbols). Asterisks indicate the significance ($P \leqslant 0.05$) of the frequency-dependent changes. Error bars that fall within the symbols are not shown.

concentration tested (50 μM), decreased dP/dt_{max} by 50–60%. The use of higher concentrations was prevented by drug solubility.

To assess the relative selectivities of norbormide and verapamil for contractility and AV conduction, the effects of each drug on AV conduction and dP/dt_{max} at the two frequencies employed were correlated. Figure 6 shows that, for any AV conduction change considered, norbormide depressed heart contractility less than verapamil. Furthermore, while the curves correlating the mechanical and electrical effects of verapamil at 2.5 Hz and 3.3 Hz were clearly separate, those of norbormide were apparently coincident, indicating that the mechanical effects of norbormide have the same dependence on frequency as the electrical effects.

Spontaneously beating right atrium

In control conditions, spontaneous right atrium frequency was 167 ± 23 beats min^{-1} ($n=5$). Norbormide, as well as verapamil, decreased spontaneous frequency in a concentration-dependent way (Figure 7). At the highest concentration tested (50 μM) norbormide completely stopped sinusal activity in 4 out of 5 experiments. The relative ability of the two drugs to decrease sinus rate and AV conduction velocity was compared by correlating the magnitude of the chronotropic effects on right atrium and that of the dromotropic effects on isolated heart (driven at 2.5 Hz). Data analysis showed that at any concentration considered norbormide, at an equivalent effect on the AV node, exerted a greater effect than verapamil on sinus node (Figure 8).

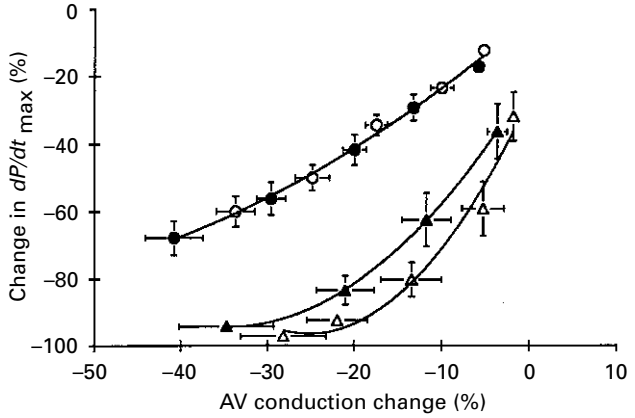

Figure 6 Relationships between the inotropic and the dromotropic effects of norbormide (○, ●) and verapamil (△, ▲), induced in isolated hearts driven at 2.5 Hz (open symbols) and 3.3 Hz (solid symbols). Each point represents the average inotropic and dromotropic effects simultaneously measured on the same heart ($n=5$). Error bars that fall within the symbols are not shown.

Discussion

In agreement with previous results obtained in guinea-pig arteries (Bova *et al.*, 1996), the present work on guinea-pig car-

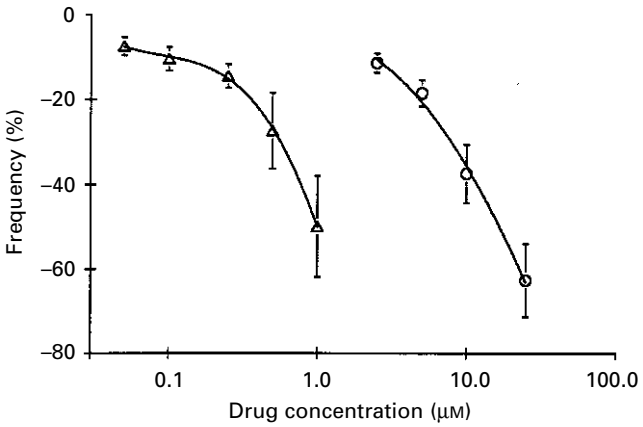

Figure 7 Concentration response curves for verapamil (△) ($n=5$) and norbormide (○) ($n=5$) for their negative chronotropic effects on spontaneously beating right atria.

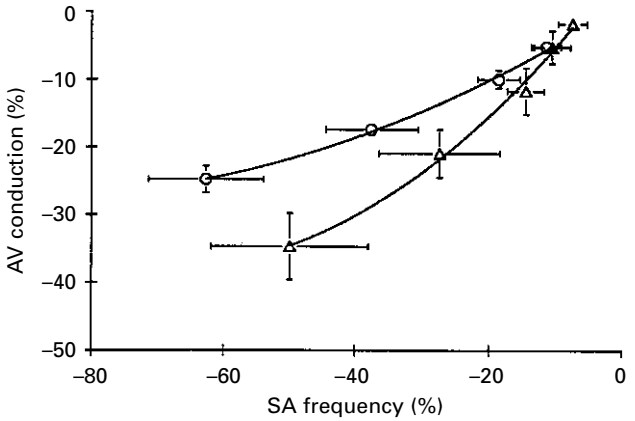

Figure 8 Relationships between the chronotropic and the dromotropic effects of norbormide (○) and verapamil (△). For each drug, the concentrations inducing the effects on AV node and SA node were the same. Each point represents the average chronotropic and dromotropic effects induced on spontaneously beating right atria and isolated hearts ($n=5$) paced at 2.5 Hz, respectively.

diac tissues and myocytes shows that norbormide has a pharmacodynamic profile fully consistent with Ca-antagonist activity and that it shares many of the properties of verapamil. In fact, in isolated heart preparations both drugs produced negative inotropic and chronotropic effects, depressed AV conduction and dilated coronary vessels. Such properties are common to all the calcium-antagonists, although the potency of their effects on force of contraction, sinus, rate, AV conduction, and coronary tone may vary markedly (Godfraind *et al*., 1986).

Evidence that the mechanism of action of norbormide is a block of calcium channels was provided by the experiments in single ventricular myocytes. The inhibition of L-type calcium current induced by norbormide was in some respects similar to that induced by verapamil. First of all, neither norbormide nor verapamil shifted the current voltage relationship along the voltage axis. Secondly, the effect of either drugs on the calcium current was strongly use-dependent, as shown by the lack of effect in the absence of cell stimulation and by the cumulative current decrease under repeated depolarizations. According to the receptor-modulated hypothesis, the enhanced block with repeated depolarization can be explained by a higher affinity of the drug to open and/or inactivated channels than to resting channels (Hondeghem & Katzung, 1977). Various degrees of use-dependence have also been reported for other calcium-antagonists. In a comparative study on the electrophysiological properties of D 600 (a methoxy-derivative of verapamil), diltiazem and nitrendipine, Lee & Tsien (1983) found that D 600 had the greatest use-dependence and nitrendipine the lowest, while diltiazem was in between. Another study (Uehara & Hume, 1985) confirmed these results with D 600 and diltiazem and found that nifedipine, like nitrendipine, had negligible use-dependence. Thus, in this respect, norbormide behaves differently from the dihydropyridines and similarly to verapamil and diltiazem. However, the electrophysiological behaviours of norbormide and verapamil are not completely overlapping, a notable difference existing with respect to the time-course of the onset of channel block during repeated depolarizations. The time constant of the process was, in fact, about 4 times smaller for norbormide (23 s) than for verapamil (91 s), indicating a faster kinetics of the interaction of norbormide with the calcium channel. The kinetics of interaction with the channel determines the frequency-blockade relationship in that the frequency at which channel block appears (threshold) and plateaus is higher for drugs with fast kinetics than for drugs with slow kinetics (Weirich & Antoni, 1990).

The voltage-clamp data obtained in single ventricular myocytes correlated well with the negative inotropic effect induced by norbormide in the whole heart. This effect has been observed with all the calcium-antagonists, although to different degrees (Godfraind *et al*., 1986). It has been found that equimolar concentrations of nifedipine, verapamil and diltiazem induced a negative inotropic effect that was greatest for nifedipine and lowest for diltiazem (Church & Zsoter, 1980). Since norbormide is about 100 times less potent than verapamil in depressing guinea pig left ventricle function, it seems more similar in this respect to diltiazem. In agreement with the use-dependence of the effect on I_{Ca}, the negative inotropic effect induced by norbormide is frequency-dependent. The observation that the negative inotropic effect of verapamil did not significantly increase when the driving rate was increased from 2.5 to 3.3 Hz suggests that, owing to the slow kinetics of interaction with the calcium channel, in this range of frequencies the frequency-block relationship is close to saturation (see above).

Like verapamil, norbormide exhibited marked electrophysiological effects on the SA node (bradycardia) and on the AV node (decrease in conduction velocity). However, compared to verapamil, norbormide depressed sinus frequency more than AV conduction, and AV conduction more than ventricular contractility. This pattern of action indicates that norbormide, compared to verapamil, has greater selectivity for the nodal tissues than for ventricular myocardium. Theoretically, this property could represent a therapeutic advantage in terms of drug safety in patients with reduced heart contractility being treated for supraventricular tachyarrhythmias. Furthermore, analysis of the relationship between drug-induced changes in AV conduction and ventricular contractility shows that the two effects are approximately linearly correlated and in the case of norbormide have the same degree of rate-dependence. On the basis of this functional difference it can be hypothesised that the two drugs bind to different sites in the channel or, alternatively, that verapamil has an additional mechanism(s) of action other than Ca channel blockade. Although intracellular effects have been obtained for verapamil, they occur at exceedingly high concentrations (Colvin *et al*., 1982; Wang *et al*., 1984), making the latter hypothesis rather unlikely.

Similar to class 1 antiarrhythmic agents, norbormide exhibited a weak but definite rate-dependent effect on intraventricular conduction time (QRS enlargement), while, in the range of concentrations tested, verapamil did not modify the QRS interval. It is known that the (+) isomer of verapamil, at concentrations higher than those employed in our study, blocks Na channels (Bayer *et al*., 1975). Since norbormide is also a racemic mixture of 5 stereoisomers (Poos *et al*., 1965), it is possible that its effects on intraventricular conduction are produced by different isomers blocking Na channels. No data are at present available on the activities of the individual isomers of norbormide.

The vasorelaxant effect of norbormide, previously shown in systemic vessels of guinea-pig, was also present on the coronary vascular bed and was roughly equivalent to that of verapamil. However, it should be considered that, since coronary tone in isolated hearts is largely dependent on myocardial contractility (Feigl, 1983), the direct coronary effects of drugs with different negative inotropic effects cannot be reliably compared.

In conclusion, the overall pharmacological profile outlined by our findings indicates that norbormide is a Ca-antagonist with marked cardiac electrophysiological effects. The comparison with verapamil points out some differences concerning the balance between the electrical and mechanical effects and the degree of their dependence on frequency. In addition, since the chemical structure of norbormide is unrelated to that of any known Ca-antagonists, it is conceivable that it may represent the prototype of a new class of Ca-antagonists. More experimental work is needed to identify the binding site of norbormide on the Ca channel and to define the pharmacological activity of the individual stereoisomers.

The authors wish to thank Mr G. Greggio for skilful technical assistance and I.N.D.I.A. Industrie Chimiche S.p.A. (Padova, Italy) for the kind gift of norbormide. This work was supported by grants from CNR (92.02194 CT14, FATMA 93.00655 PF41) and MURST (40% and 60%).

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(Received May 20, 1996
Revised September 15, 1996
Accepted September 27, 1996)