Stereoselective effects of the enantiomers of bupivacaine on the electrophysiological properties of the guinea-pig papillary muscle

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1 Direct myocardial effects of the S(–)- and R(+)-enantiomers of bupivacaine were compared in the guinea-pig isolated papillary muscle by recording transmembrane action potentials with the standard microelectrode technique.

2 In 5.4 mM K⁺, at a stimulation rate of 1 Hz, the maximal rate of depolarization ($\dot{V}_{max}$) was reduced to $59.9 \pm 1.4\%$ ($n = 10$) of control (mean $\pm$ s.e. mean) in the presence of 10 μ M R(+)-bupivacaine, and to $76.7 \pm 1.2\%$ ($n = 14$) in the presence of the same concentration of S(–)-bupivacaine. This was mainly due to a difference in time constant at which block dissipated during the diastolic period. Recovery was slower in the presence of R(+)-bupivacaine. The slower recovery in the presence of R(+)-bupivacaine resulted also in a more pronounced frequency-dependent block of $\dot{V}_{max}$.

3 Time constants for recovery from use-dependent block became significantly faster for both enantiomers on hyperpolarization, while no significant change was observed at depolarization. At all membrane potentials recovery was slower in the presence of R(+)-bupivacaine.

4 The action potential duration (APD) was shortened to a greater extent in the presence of R(+)-bupivacaine over a large range of stimulation frequencies.

5 We conclude that S(–)-bupivacaine affects $\dot{V}_{max}$ and APD in the guinea-pig papillary muscle less than the R(+)-enantiomer at different rates of stimulation and resting membrane potentials.

Keywords: Bupivacaine; enantiomers; stereoselectivity; heart; guinea-pig papillary muscle; standard microelectrode technique; maximal rate of depolarization; $\dot{V}_{max}$

Introduction

Bupivacaine (Marcaine) is a commonly used drug for regional anaesthesia. During the last decade, several reports described cases of severe, even fatal, cardiotoxicity (sudden cardiovascular collapse) after presumed accidental intravascular injection of bupivacaine (Albright, 1979), or after administration of large overdoses of bupivacaine (Covino, 1986).

The mechanism of the cardiotoxicity of bupivacaine is complex. In several studies the observed cardiotoxicity was not due to a direct effect on the heart, but indirectly to a blockage of the sympathetic cardiac innervation (Hotvedt *et al.*, 1983), or to a central nervous system-mediated mechanism with potentially fatal arrhythmias (Thomas *et al.*, 1986). In many other studies *in vivo* (by injection *in situ*; intracoronary, intraatrial) and *in vitro*, a direct cardiotoxicity was shown with conductance disturbances and severe negative inotropic effects. In several studies, animals died most often of ventricular arrhythmias or ventricular fibrillation preceded by a progressive widening of the QRS interval (Kotelko *et al.*, 1984; Hotvedt *et al.*, 1985; Rosen *et al.*, 1985; Nath *et al.*, 1986). But in some other studies, animals died in complete cardio-depression without rhythm disturbances (Liu *et al.*, 1982).

Numerous papers have shown that bupivacaine is much more cardiotoxic than lignocaine. The ratio of the potencies to depress cardiac contractility by bupivacaine and lignocaine is analogous to the anaesthetic potency ratio (*in vivo* 4–5 : 1) (Nath *et al.*, 1986; Buffington, 1989) and this ratio is too small to explain the observed large differences in cardiotoxicity. The much larger potency ratio required to depress cardiac conduction (16 : 1 to affect seriously QRS or PR conduction times and cause severe dysrhythmias like ventricular fibrillation) seems to be the primary mechanism (Komai & Rusy, 1981; Nath *et al.*, 1986). Bupivacaine is much more arrhythmogenic than lignocaine. Bupivacaine has been shown to cause Pur-

kinje fibre-ventricular muscle conduction block in rabbit heart preparations *in vitro* at a concentration of 10–15 μ M (Moller & Covino, 1988).

Clinically used bupivacaine is a racemic mixture of S(–)-bupivacaine and R(+)-bupivacaine. Previous studies (Ludueno *et al.*, 1972; Aberg, 1972; Aps & Reynolds, 1978) demonstrated that the potency and duration of the anaesthesia *in vivo* were equal or in some cases larger for S(+)-bupivacaine than for the R(+)-enantiomer. Furthermore S(–)-bupivacaine was less toxic than R(+)-bupivacaine: the LD₅₀ was about 30 to 40% lower for R(+)-bupivacaine when administered intravenously in mice, rat, and rabbit or subcutaneously in mice and rat (Ludueno *et al.*, 1972; Aberg, 1972). The mechanism of the observed difference in the *in vivo* toxicity of the enantiomers of bupivacaine has never been examined. As stereoselective effects of class I antiarrhythmics, which are also local anaesthetics, have been reported (Carmeliet *et al.*, 1986; Block *et al.*, 1988; Hill *et al.*, 1988), we have explored whether the observed difference in the *in vivo* toxicity between the two enantiomers could be attributed to a different potency on the heart. Since the cardiotoxicity of bupivacaine seems to be mainly of electrophysiological origin, we have studied the effects of its enantiomers on the electrophysiological properties of guinea-pig isolated papillary muscle at different membrane potentials and stimulation frequencies.

Methods

Guinea-pigs (300–500 g) of either sex were killed by a blow on the head, the hearts were quickly removed and transferred to Tyrode solution of the following composition (mM): NaCl 127, NaHCO₃ 23.8, KCl 5.4, MgCl₂ 0.5, CaCl₂ 1.8 and glucose 5. The solution was gassed with 95% O₂ and 5% CO₂ and had a pH of 7.4. Papillary muscles of the right ventricle were dissected and fixed to the bottom of a perfusion bath by thin

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cotton threads. The bath (volume 0.25 ml) was perfused with Tyrode solution at a constant rate (2.7 ml min^{-1}). The preparation was allowed to recover for 2 h during which stimuli (0.3 ms in duration; intensity: 1.5 times threshold) were applied at a rate of 1 Hz via bipolar Ag–AgCl electrodes by a programmable stimulator. Transmembrane potentials were recorded with an intracellular and extracellular microelectrode (filled with 3 M KCl, resistance $1.0\text{--}1.5 \times 10^7 \text{ Ohm}$), and displayed on a Tektronix 5441 storage oscilloscope. The maximal rate of depolarization ($\dot{V}_{\max}$) was measured by electronic differentiation with a bandwidth from 0 to 10 kHz. The action potential duration (APD) was measured directly from the oscilloscope by extrapolating the terminal slope of the action potential to the resting membrane potential. APD_{90} and APD_{50} are the APD after 90 and 50% repolarization, respectively.

In all experiments the preparation was stimulated at 1 Hz and perfused with normal Tyrode solution, containing 5.4 mM external K^+ unless otherwise stipulated. The experiments were only evaluated when the microelectrode impalement was stable during the whole duration of the experiment and when $\dot{V}_{\max}$ did not change with the stimulation intensity or duration.

The enantiomers of bupivacaine HCl were kindly provided by Astra Belgium. Concentrated stock solutions were made in twice distilled water and kept in a freezer at -20°C . Drug solutions were made as needed by appropriate dilution.

Concentration-effect relationships were recorded by increasing the drug concentration stepwise from $0.3 \mu\text{M}$ to $80 \mu\text{M}$. Each concentration was applied for 15 min and the diverse action potential parameters were measured at the end of these periods.

Tonic block was defined as the percentage decrease of the first $\dot{V}_{\max}$ after a long period of rest (5 min) in presence of the drug, as compared with the control in the same conditions. Phasic block was defined as the further decrease in $\dot{V}_{\max}$ as stimulation was continued at 1 Hz, calculated relative to the first $\dot{V}_{\max}$ in control conditions.

To record the recovery process of $\dot{V}_{\max}$, the preparation was stimulated at a rate of 60 min^{-1} . Rest periods of increasing duration were applied, after which the preparation was again stimulated at 1 Hz until steady state. The $\dot{V}_{\max}$ of the first action potential after each rest period was noted as a function of the time of rest. The rest period was gradually increased until no further increase of the first $\dot{V}_{\max}$ after the rest period could be obtained. For recovery periods shorter than 1 s a method with extrapulses was used. Every 15th pulse an extra-stimulus was applied, first as close as possible to the preceding action potential, then the coupling interval gradually was increased. The $\dot{V}_{\max}$ of the extra evoked action potential was noted as a function of the coupling interval.

Inactivation curves were obtained by increasing stepwise the external K^+ concentration from 2.7 mM to 21 mM with exactly 5 min interval and noting $\dot{V}_{\max}$ as a function of the resting membrane potential.

Results were fitted by non linear regression: using the sum of 2 exponentials for recovery experiments, and the Boltzmann equation for inactivation curves. Results in tables and figures are indicated by mean $\pm$ standard error of mean (s.e.mean). Statistics were calculated by the two tailed Student's *t* test; $P < 0.05$ was considered as statistically significant. When more than two means had to be compared we made use of analysis of variance (ANOVA). ANOVA was followed by multiple pairwise comparisons, based on Bonferroni's *t* test where the significance level is set at $P < 0.05/m$ (where *m* = the number of simultaneous comparisons).

Results

General description of the effects on the action potential

Typical action potential recordings in the presence of $10 \mu\text{M}$ S(–)-bupivacaine (a) and R(+)-bupivacaine (b) are shown in Figure 1. Both enantiomers caused a reduction of the maximal

Figure 1 Action potential and $\dot{V}_{\max}$ in control conditions and 45 min after superfusion with the 2 enantiomers of bupivacaine $10 \mu\text{M}$. The guinea-pig papillary muscle was stimulated at 1 Hz. External K^+ was 5.4 mM. Both enantiomers reduced $\dot{V}_{\max}$ and shortened the action potential. The maximal rate of depolarization was clearly more reduced by the R(+)-enantiomer of bupivacaine.

rate of rise ($\dot{V}_{\max}$), action potential amplitude (APA) and action potential duration (APD). No effect on the resting membrane potential was observed. The reduction of $\dot{V}_{\max}$ as well as APD was more pronounced with R(+)-bupivacaine than with S(–)-bupivacaine.

Concentration-effect relationships Concentration-effect curves for the different parameters were made at a stimulation frequency of 1 Hz. Although the number of experiments was restricted, the concentration-effect curve for $\dot{V}_{\max}$ (Figure 2) showed statistically significant differences between the two enantiomers at all concentrations; R(+)-bupivacaine caused a more marked reduction of $\dot{V}_{\max}$ than S(–)-bupivacaine. The concentration-effect curve for R(+)-bupivacaine had a mean K_d value of $16.1 \pm 0.2 \mu\text{M}$ and a Hill coefficient of 0.86 ± 0.06 ($n = 3$) while for S(–)-bupivacaine we obtained a K_d value of $38.7 \pm 2.7 \mu\text{M}$ and a Hill coefficient of 1.04 ± 0.05 ($n = 4$).

The effect of the drug on APD was less pronounced. At a concentration of $80 \mu\text{M}$, the APD was reduced to about 70% for both enantiomers. The concentration-effect curve for the APD showed no statistically significant differences between the two enantiomers. For the rest of the experiments a concentration of $10 \mu\text{M}$ was chosen.

Steady state effects of $10 \mu\text{M}$ bupivacaine To obtain steady state values a wash-in period of 45 min was needed. Table 1 shows the mean steady state values for the different action potential parameters in control and in the presence of either enantiomer.

At a concentration of $10 \mu\text{M}$ and a stimulation frequency of 1 Hz, $\dot{V}_{\max}$ was about 77% of control in the presence of S(–)-bupivacaine while $\dot{V}_{\max}$ amounted to about 60% in the presence of R(+)-bupivacaine. The difference in potency was statistically significant.

We also observed statistically significant differences in effect on the action potential amplitude (APA), which was also more depressed in the presence of the dextrorotatory isomer of bupivacaine.

In contrast to the results obtained after 15 min perfusion (concentration-effect curves, Figure 2b), the APD and APD_{90} after 45 min perfusion were significantly more shortened with R(+)-bupivacaine than in the presence of S(–)-bupivacaine (two-tailed Student's unpaired *t* test). In the presence of R(+)-

Figure 2 $\dot{V}_{max}$ (a) and APD (b) relative to the control as a function of the concentration of R(+)-bupivacaine (○, $n = 3$) and S(-)-bupivacaine (●, $n = 4$) in guinea-pig papillary muscles. The preparation was stimulated at 1 Hz in 5.4 mM K^+ . The control $\dot{V}_{max}$ and APD were $345 \pm 17 \text{ V s}^{-1}$ and $169 \pm 8 \text{ ms}$ for R(+)-bupivacaine, and $330 \pm 1 \text{ V s}^{-1}$ and $171 \pm 5 \text{ ms}$ for the S(-)-enantiomer. S.e.mean are indicated by vertical bars when they are larger than the symbols. Statistical significance of the difference between the 2 enantiomers is indicated by asterisks: * $P < 0.05$; ** $P < 0.01$.

bupivacaine APD was reduced to $76.5 \pm 2.5\%$ ($n = 11$) of control and to $83.4 \pm 1.5\%$ ($n = 14$) in the presence of S(-)-bupivacaine.

Tonic and phasic block of $\dot{V}_{max}$ At a stimulation frequency of 1 Hz and an external potassium concentration of 5.4 mM, the total block of $\dot{V}_{max}$ consisted of tonic and use-dependent block.

In these conditions phasic block was more pronounced than tonic block. Tonic block was $7.7 \pm 0.8\%$ ($n = 5$) for S(-)-bupivacaine and $10.7 \pm 1.9\%$ ($n = 5$) for the R(+)-enantiomer, results that were not statistically significantly different. Tonic block disappeared with hyperpolarization (2.7 mM K^+) and increased with depolarization (8.1 mM K^+). In 2.7 mM K^+ , tonic block was $-1.9 \pm 1.6\%$ ($n = 4$) for S(-)-bupivacaine and $2.8 \pm 2.8\%$ ($n = 2$) for R(+)-bupivacaine; in 8.1 mM K^+ , tonic block increased to $34 \pm 3\%$ ($n = 3$) and $47 \pm 2\%$ ($n = 2$), respectively.

In 5.4 mM K^+ phasic block was larger for R(+)-bupivacaine [$30.3 \pm 1.6\%$ ($n = 5$)] than for S(-)-bupivacaine [$16.5 \pm 1.3\%$ ($n = 5$)], which was statistically significant.

In order to examine further the difference in potency, time-dependent and potential-dependent characteristics of both drugs were studied.

Frequency and time-dependent effects

$\dot{V}_{max}$ -stimulation interval relationship Figure 3(a) shows the relationship between $\dot{V}_{max}$ and the stimulation interval. In control conditions, diminishing the stimulation interval from 10 s to 0.25 s caused a 10% decrease of $\dot{V}_{max}$, presumably due to a slow component in the inactivation of the sodium current. Bupivacaine reduced the $\dot{V}_{max}$ at all stimulation intervals. At an interval of 10 s, $\dot{V}_{max}$ was on average $85.4 \pm 1.7\%$ ($n = 5$) for S(-)-bupivacaine and $82.6 \pm 1.4\%$ ($n = 5$) for R(+)-bupivacaine when compared with the control. The reduction of $\dot{V}_{max}$ was quite constant at stimulation intervals between 2 to 10 s in the presence of S(-)-bupivacaine and between 4 to 10 s in the presence of R(+)-bupivacaine. Only when the stimulation interval was diminished from 2 to 0.25 s did the effect on $\dot{V}_{max}$ become more pronounced. At an interval of 0.25 s, $\dot{V}_{max}$ was decreased to about $29.0 \pm 0.9\%$ ($n = 5$) for S(-)-bupivacaine and to about $16.9 \pm 1.8\%$ ($n = 5$) for R(+)-bupivacaine compared to the control at a stimulation interval of 10 s. The difference in block between the two enantiomers became relatively larger as the stimulation interval was shortened: at an interval of 0.25 s, $\dot{V}_{max}$ in the presence of R(+)-bupivacaine was 1/5 of the $\dot{V}_{max}$ at an interval of 10 s, while in presence of the S(-)-enantiomer this ratio was only 1/3.

The difference in effect between the two enantiomers was statistically significant for stimulation intervals between 2 s and 0.25 s (frequencies between 30 min^{-1} and 240 min^{-1}). At longer stimulation intervals this difference was not statistically significant. These experiments confirm that in 5.4 mM K^+ the difference in effect of the 2 enantiomers on $\dot{V}_{max}$ is due to a

Table 1 Steady state effects of bupivacaine (10 μM)

	$\dot{V}_{max}$ (V s^{-1})		APA (mV)		Em (mV)	
	mean $\pm$ s.e.mean	(n)	mean $\pm$ s.e.mean	(n)	mean $\pm$ s.e.mean	(n)
Control	329 ± 12	(14)	127.5 ± 0.3	(14)	-88.7 ± 0.7	(14)
S(-)-bupivacaine	253 ± 12	(14)	124.8 ± 0.3	(14)	-88.6 ± 0.7	(14)
%	$76.7 \pm 1.2^{**}$	(14)	$97.9 \pm 0.2^*$	(14)		
Control	320 ± 15	(11)	126.4 ± 0.5	(11)	-88.7 ± 0.8	(11)
R(+)-bupivacaine	193 ± 9	(10)	122.7 ± 0.6	(11)	-88.6 ± 0.8	(11)
%	$59.9 \pm 1.4^{**}$	(10)	$97.1 \pm 0.3^*$	(11)		
	APD (ms)		APD ₉₀ (ms)		APD ₅₀ (ms)	
	mean $\pm$ s.e.mean	(n)	mean $\pm$ s.e.mean	(n)	mean $\pm$ s.e.mean	(n)
Control	180 ± 4	(14)	173 ± 4	(14)	150 ± 4	(14)
S(-)-bupivacaine	150 ± 4	(14)	142 ± 5	(14)	115 ± 4	(14)
%	$83.4 \pm 1.5^*$	(14)	$82.0 \pm 1.6^*$	(14)	76.2 ± 1.7	(14)
Control	180 ± 5	(11)	173 ± 5	(11)	148 ± 4	(11)
R(+)-bupivacaine	137 ± 4	(11)	129 ± 4	(11)	104 ± 4	(11)
%	$76.5 \pm 2.5^*$	(11)	$75.1 \pm 2.5^*$	(11)	70.9 ± 2.8	(11)

Stimulation frequency: 60 min^{-1} . $[\text{K}^+]_o = 5.4 \text{ mM}$

Student's unpaired t test between S(-) and R(+)-bupivacaine: * $P < 0.05$; ** $P < 0.001$.

Figure 3 Effect of stimulation interval on $\dot{V}_{max}$ (a) and APD (b) in guinea-pig papillary muscles. Relative change in $\dot{V}_{max}$ and APD as a function of the stimulation interval in control conditions (curve without symbols), and in the presence of $10 \mu\text{M}$ R(+)-bupivacaine (○) and $10 \mu\text{M}$ S(-)-bupivacaine (●). The preparation was bathed in 5.4 mM K^+ . The control value at a stimulation interval of 10 s was taken as $100\% : 313 \pm 6 \text{ V s}^{-1}$ and $178 \pm 6 \text{ ms}$ for R(+)-bupivacaine, $322 \pm 18 \text{ V s}^{-1}$ and $176 \pm 7 \text{ ms}$ for S(-)-bupivacaine. The number of experiments was 5 for both enantiomers for the effect on $\dot{V}_{max}$ and 4 for the effect on the APD. S.e.mean are indicated by vertical bars when they are larger than the symbols. Repeated measures ANOVA revealed significant differences between the two enantiomers both for $\dot{V}_{max}$ and the APD. Statistical significance of the difference between the two enantiomers is indicated with asterisks; * $P < 0.05$; ** $P < 0.01$ (Student's t test).

difference in use-dependent block rather than a difference in tonic block.

APD-stimulation interval relationship Figure 3(b) shows the effect of the two enantiomers of bupivacaine on the action potential duration at different stimulation intervals. A step-wise decrease in the interval from 10 s to 0.25 s in control conditions caused a gradual shortening of the APD to about 66% of its value at 10 s. At a stimulation interval of 10 s, APD was $87.8 \pm 1.3\%$ ($n = 4$) of the control value in the presence of S(-)-bupivacaine and $79.3 \pm 1.0\%$ ($n = 4$) in the presence of R(+)-bupivacaine. At stimulation intervals from 10 to 0.4 s, both enantiomers decreased APD. At intervals of 0.3 and 0.25 s, the APD in the presence of S(-)-bupivacaine and R(+)-bupivacaine was not significantly different from the one obtained in control conditions (two tailed Student's paired t test). At a stimulation interval of 0.25 s, APD was $103.5 \pm 1.0\%$ ($n = 3$) of the control for S(-)-bupivacaine and $96.7 \pm 5.4\%$ ($n = 3$) for R(+)-bupivacaine. The action potential duration appeared to be less affected at short stimulation intervals than at long stimulation intervals.

At all intervals the action potential duration was less in the presence of R(+)-bupivacaine than in the presence of S(-)-bupivacaine but the difference was only statistically significant at stimulation intervals greater than 0.4 s.

Onset and recovery kinetics off/from use-dependent block of $\dot{V}_{max}$

Onset kinetics: When the preparation was stimulated at a frequency of 1 Hz in 5.4 and 8.1 mM K^+ after a long period of rest (5 min), the $\dot{V}_{max}$ of the first action potential was smaller in presence of the drug than in control conditions (cf. Figure 4). For the second action potential, $\dot{V}_{max}$ was much lower than the first for both enantiomers of bupivacaine and continued stimulation did not cause a significant further decrease of $\dot{V}_{max}$. Therefore the rate at which the use-dependent block developed after a rest period was too fast to be fitted. We did not record onset at higher rates of stimulation.

Recovery kinetics: The kinetics at which $\dot{V}_{max}$ reprimed during a rest period could be fitted as a sum of two exponentials; a fast one representing the recovery of the drug free channels, and a slower one representing the recovery of the drug occupied channels. Figure 4 (right panels) shows typical recovery patterns for S(-)-bupivacaine and R(+)-bupivacaine at different external K^+ concentrations. Table 2 summarizes the fast and slow recovery time constants at different external

Figure 4 Onset of rate-dependent block of $\dot{V}_{max}$ in control conditions (left), and onset and recovery (middle and right panel) in presence of $10 \mu\text{M}$ S(-)-bupivacaine (a) and R(+)-bupivacaine (b) in different external K^+ concentrations for a typical experiment on guinea-pig papillary muscles. For onset, $\dot{V}_{max}$ is plotted as a function of time when the preparation is stimulated after 5 min of rest. For recovery, rest periods of variable duration were applied and the $\dot{V}_{max}$ of the first action potential after each period of rest is plotted as a function of the time of rest. Basic frequency was 1 Hz. Tonic block was larger and recovery slower for the R(+)-enantiomer. Tonic block increased with depolarization for both enantiomers.

Table 2 Recovery time constants of $\dot{V}_{max}$ at different K^+ -concentrations

	K^+ 2.7 mM		K^+ 5.4 mM		K^+ 8.1 mM	
	mean $\pm$ s.e.mean	(n)	mean $\pm$ s.e.mean	(n)	mean $\pm$ s.e.mean	(n)
S(-)-bupivacaine						
fast (ms)	30 $\pm$ 11	(3)	19 $\pm$ 5	(5)	25 $\pm$ 2	(4)
slow (ms)	365 $\pm$ 19*	(5)	560 $\pm$ 28**	(8)	533 $\pm$ 20**	(5)
R(+)-bupivacaine						
fast (ms)	8	(1)	14 $\pm$ 2	(4)	30	(1)
slow (ms)	585 $\pm$ 83*	(3)	981 $\pm$ 86**	(7)	842 $\pm$ 42**	(4)

Concentration bupivacaine: 10 μ M. Basic frequency: 60 min⁻¹.

Repeated measures ANOVA on time constants obtained in experiments where effects of differing K^+ concentration were measured in the same preparation, revealed significant differences between enantiomers and significant effects of changing K^+ concentration. Comparison between S(-)- and R(+)-bupivacaine: * $P < 0.05$; ** $P < 0.001$ (Student's t test). Multiple pairwise comparisons between time constants in the different K^+ concentrations revealed that recovery in 2.7 mM K^+ was faster than in 5.4 mM K^+ for each enantiomer (Bonferroni t test).

K^+ concentrations. At all K^+ levels, recovery of $\dot{V}_{max}$ was significantly slower in the presence of R(+)-bupivacaine than in the presence of S(-)-bupivacaine. In 5.4 mM K^+ , the slow phase had a time constant of about 981 ms in the presence of R(+)-bupivacaine against about 560 ms for S(-)-bupivacaine.

When hyperpolarizing the membrane by superfusion with 2.7 mM $[K^+]_o$, the recovery became significantly faster ($\tau = 585$ ms for R(+)-bupivacaine and 365 ms for S(-)-bupivacaine) while no significant change was observed during depolarization with 8.1 mM $[K^+]_o$ (see Figure 4).

Figure 5 Inactivation curve of $\dot{V}_{max}$ in guinea-pig papillary muscle. Typical inactivation curves at a frequency of 0.8 min⁻¹ in control conditions ($\blacktriangle$), in the presence of 10 μ M S(-)-bupivacaine (a, $\bullet$) and in the presence of 10 μ M R(+)-bupivacaine (b, $\blacksquare$). In the right part of the figure, inactivation curves are normalized. In presence of the drug, $\dot{V}_{max}$ was depressed at all resting membrane potentials except at very negative potentials, and the inactivation curve was shifted to more negative membrane potentials. Inactivation curves were more shifted by the R(+)-enantiomer.

Voltage dependence of the effects on $\dot{V}_{max}$

Figure 4 showed an increase of tonic block with depolarization, suggesting voltage-dependence for both enantiomers. The voltage-dependence of the effect on $\dot{V}_{max}$ was investigated by recording inactivation curves.

To avoid time-dependent effects, a very low stimulation frequency (0.8 min⁻¹) was applied. Figure 5 shows typical inactivation curves. At all potentials the $\dot{V}_{max}$ was depressed except at very negative membrane potentials; in 2.7 mM K^+ , $\dot{V}_{max}$ was 98.6 $\pm$ 1.9% ($n = 4$) of the control for S(-)-bupivacaine and 99.2 $\pm$ 1.8% ($n = 3$) for R(+)-bupivacaine.

Both enantiomers shifted the inactivation curves to more negative potentials. R(+)-bupivacaine shifted the half maximal potential to -11.1 $\pm$ 0.7 mV ($n = 3$) to more negative potentials, while S(-)-bupivacaine caused a shift of -8.1 $\pm$ 0.5 mV ($n = 4$). The difference between the two enantiomers was statistically significant. There was no statistically significant difference between the two enantiomers in the slope of the inactivation curves.

Discussion

The present study provides strong evidence that the interaction of bupivacaine with cardiac cell membranes is dependent upon the stereochemical conformation of the drug. R(+)-bupivacaine causes a stronger reduction of $\dot{V}_{max}$ at different stimulation rates and resting membrane potentials. This could be at the basis of the difference in lethal toxicity between R(+)-bupivacaine and the S(+)-enantiomer, previously reported (Ludueno *et al.*, 1972; Aberg, 1972).

A mean apparent dissociation constant, K_d of 16 μ M was found for the reduction of $\dot{V}_{max}$ at 1 Hz by R(+)-bupivacaine versus a K_d of 39 μ M for S(-)-bupivacaine. These K_d values are very similar to the dissociation constants calculated by Leeson *et al.* (1989) for the inhibition of the veratridine effect on frog sciatic nerve and the reduction of I_{Na} in nerves (12 μ M for R(+)-bupivacaine and 42 μ M for S(-)-bupivacaine). Conversely, the anaesthetic potency *in vivo* of S(-)-bupivacaine seems to be larger or equal (Ludueno *et al.*, 1972). Another study on isolated frog nerves (Aberg, 1972), reported no difference in nerve-blocking effect between the two enantiomers.

In 5.4 mM K^+ , both enantiomers caused tonic block and a marked use-dependent block developed at high stimulation frequencies. Since hyperpolarization completely relieved tonic block in the presence of 10 μ M of either enantiomer of bupivacaine, both enantiomers must have a small affinity for channels in the rested state. Since depolarization strongly increased tonic block, we conclude that the observed tonic block was due to binding to the inactivated state (I-block). The marked shift of the inactivation curves at a frequency where all use-

dependent effects are absent, also reveals the I-block. This is in agreement with the results of Clarkson & Hondeghem (1985) who showed in voltage-clamped guinea-pig papillary muscle that bupivacaine is mainly an inactivated state blocker with low affinity for rested and activated sodium channels.

The affinity of the inactivated channel appears to be larger for R(+)-bupivacaine than for S(-)-bupivacaine since the shift of the inactivation curves was more pronounced in the presence of R(+)-bupivacaine. The difference in shift was less striking than the one we obtained with the enantiomers of tocainide (Carmeliet *et al.*, 1986).

At 1 Hz and 5.4 mM K⁺, an important amount of use-dependent block was present. Use-dependent block of $\dot{V}_{max}$ results from block of Na⁺ channels during the action potential and incomplete unblocking during the diastolic period. R(+)-bupivacaine unblocked significantly slower than did S(-)-bupivacaine at all tested membrane potentials. In 5.4 mM K⁺, the time constant for recovery from use-dependent block was 560 ms for S(-)-bupivacaine and 981 ms for R(+)-bupivacaine. This is in agreement with the absence of use-dependent block of $\dot{V}_{max}$ at stimulation intervals larger than 2 or 4 s. The slower unblocking of R(+)-bupivacaine blocked channels explains the larger reduction of $\dot{V}_{max}$ at stimulation intervals lower or equal to 1.3 s.

When cells were hyperpolarized, recovery from use-dependent block became significantly faster, as is also the case with lignocaine. No increase of recovery time was seen upon depolarization. Faster recovery of $\dot{V}_{max}$ upon hyperpolarization could be due to unblocking via a faster transition pathway: from the drug occupied inactivated state, over the drug occupied rested state, to the drug free rested state (ID → RD → R) as suggested by Bean *et al.* (1983) for lignocaine.

In summary, R(+)-bupivacaine has a larger effect on $\dot{V}_{max}$ at different resting membrane potentials and stimulation rates than does S(-)-bupivacaine. Very recently the larger block of the Na⁺ current by R(+)-bupivacaine in guinea-pig isolated cardiac myocytes was explained by Valenzuela *et al.* (1990) as being due to differences in the degree of activation unblocking and the magnitude of the voltage shift in gating kinetics of drug associated channels. Activation unblocking would be lower and voltage shift would be larger for R(+)-bupivacaine.

As far as the action potential duration is concerned, we did not observe any significant difference in effect of the two enantiomers after 15 min; however after 45 min, when complete steady state conditions are achieved, R(+)-bupivacaine caused

a more pronounced shortening of the action potential than its enantiomer. Shortening of the action potential is presumably due to a similar mechanism as described by Carmeliet & Saikawa (1982) for lignocaine, namely inhibition of the slowly inactivating Na⁺ current. As R(+)-bupivacaine has a larger blocking effect on $\dot{V}_{max}$, which is a measure for the fast Na⁺ current, one can speculate that it has presumably also a larger effect on the slowly inactivating Na⁺ current and, as a consequence, the effect on APD would be more pronounced.

In contrast to the effect on $\dot{V}_{max}$, both enantiomers reduced the APD more at lower stimulation rates than at higher stimulation rates; at the highest frequencies the APD was not statistically different from the control APD. This finding is analogous to that of Varro *et al.* (1986) for different class I antiarrhythmic drugs. The exact reason for this phenomenon is not known. One possible explanation is that since the amplitude of the slow Na⁺ current is less pronounced at higher frequencies, the slow Na⁺ current contributes less to APD at higher frequencies and so drugs blocking this current would have less influence on APD at higher frequencies. However, block of i_K (delayed potassium current) could also play a role.

The fact that R(+)-bupivacaine has a greater effect on $\dot{V}_{max}$ and APD is possibly the explanation for the observed higher *in vivo* toxicity. Reduction of $\dot{V}_{max}$ slows conduction. Too much slowing of conduction and shortening of the APD predisposes for reentrant phenomena. Since S(-)-bupivacaine has less influence on the heart *in vitro* (present results) and has a higher LD₅₀ value, but shows a comparable anaesthetic potency *in vivo*, clinical use of only the S(-)-enantiomer of bupivacaine in analogy to ropivacaine (congener of bupivacaine and optically pure S-enantiomer) could improve the margin of safety of this widely used drug. Of course, other parameters such as pharmacokinetics and overall pharmacodynamic activity also have to be considered. Protein binding, tissue binding, clearance, uptake in heart and brain, convulsive toxicity, negative inotropic effect should all be considered for both enantiomers.

Our results with the enantiomers of bupivacaine, having identical physicochemical properties, favour the hypothesis that a specific receptor site at the sodium channel must be involved in local anaesthetic action rather than a non specific effect of disturbing the membrane.

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