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Stereospecific effects of ketamine enantiomers on canine tracheal smooth muscle

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1 Ketamine is a potent bronchodilator which relaxes airway smooth muscle (ASM). Clinically, ketamine is used as a 1:1 racemic mixture of enantiomers that differ in their analgesic and anaesthetic effects. The aim of this study was to determine whether there was a difference between the enantiomers in their ability to relax isolated ASM and to explore mechanisms responsible for any observed differences.

2 Canine tracheal smooth muscle strips were loaded with fura-2 and mounted in a photometric system to measure simultaneously force and $[Ca^{2+}]_i$. Calcium influx was estimated by use of a manganese quenching technique.

3 In strips stimulated with 0.1 μM ACh (EC_{50}) **R**(−)-ketamine (1–100 μM) caused a significantly greater concentration-dependent decrease in force ($P<0.0001$) and $[Ca^{2+}]_i$ than **S**(+)-ketamine (1–100 μM) ($P<0.0005$). In contrast, there was no significant difference between the enantiomers in their ability to inhibit calcium influx (45% decrease in influx rate for **R**(−)-ketamine and 44% for **S**(+)-ketamine, $P=0.782$). In strips contracted with 24 mM isotonic KCl (which activates voltage-operated calcium channels), the enantiomers modestly decreased force and $[Ca^{2+}]_i$; there was no significant difference between the enantiomers in their effects on force ($P=0.425$) or $[Ca^{2+}]_i$ ($P=0.604$).

4 The **R**(−)-enantiomer of ketamine is a more potent relaxant of ACh-induced ASM contraction than the S(+)-enantiomer. This difference appears to be caused by differential actions on receptor-operated calcium channels.

Keywords: Ca^{2+}-fluorescent probe fura-2; manganese quenching; ketamine and its enantiomers; lung, bronchus; bronchoconstriction; lung, trachea; canine smooth muscle; airway trachea; anaesthetic

Introduction

Ketamine is a potent bronchodilator (Huber *et al*., 1972) which relaxes airway smooth muscle (ASM) *in vitro* in part by a direct effect on ASM cells (Lundy *et al*., 1974; Hirshman *et al*., 1979; Gateau *et al*., 1989; Wilson *et al*., 1993). In a previous study we showed that ketamine relaxes canine isolated ASM contracted with the physiological agonist acetylcholine (ACh) by inhibiting calcium influx and decreasing the intracellular calcium concentration ($[Ca^{2+}]_i$) (Pabelick *et al*., 1997).

Clinically, ketamine is used as a 1:1 racemic mixture of enantiomers that differ in their analgesic and anaesthetic effects with **S**(+)-ketamine about 2–4 times more potent than the **R**(−)-ketamine (Ryder *et al*., 1978; White *et al*., 1980; 1985; Klepstad *et al*., 1990). In studies of vascular smooth muscle and guinea-pig isolated hearts (Lundy *et al*., 1986; Graf *et al*., 1995), **S**(+)-ketamine consistently produces a greater depressant effect than **R**(−)-ketamine.

Little is known about possible differences in the effects of the ketamine enantiomers on ASM (Hirota *et al*., 1996). An answer to this question would be of clinical interest. Ketamine has been recommended for the treatment of bronchospasm during general anaesthesia (Corssen *et al*., 1972). If the bronchodilating property were only due to the action of one enantiomer, treatment of bronchospasm could be made more specific. Also, many general anaesthetics are stereoselective in their actions, suggesting that they exert their primary effects at a relatively small number of sites (Franks & Lieb, 1994). Thus, any stereospecific effects of the ketamine enantiomers may provide insight into the mechanisms of action of ketamine in ASM.

The purpose of this study was to determine whether there was a difference between the two enantiomers of ketamine in their ability to relax canine ASM and to explore mechanisms responsible for any observed differences.

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Methods

Tissue preparation

Mongrel dogs of either sex were anaesthetized with an intravenous injection of pentobarbitone (30 mg kg^{-1}) and exsanguinated. A 5–10 cm portion of extrathoracic trachea was excised and immersed in chilled physiological salt solution (PSS) of the following composition (mM): NaCl 110.5, $NaHCO_3$ 25.7, dextrose 5.6, KCl 3.4, $CaCl_2$ 2.4, KH_2PO_4 1.2 and $MgSO_4$ 0.8. Fat, connective tissue and the epithelium were removed with tissue forceps and scissors.

Muscle strips (width 0.8–1.0 mm, length 4–5 mm) were mounted in a 0.1 ml quartz cuvette and were continuously superfused at a rate of 2 ml min^{-1} with PSS (37°C) aerated with 94% O_2-6% CO_2. One end of the strip was anchored via a stainless steel microforceps to a stationary metal rod; the other end was attached via a stainless steel microforceps to a calibrated force transducer (model AE801, Aksjeselkapet Mikro Elektronik). During a 2 h equilibration period, the length of the strips was increased after repeated isometric contractions (2–3 min duration) produced by 1 μM ACh until optimal length was obtained. Each strip was maintained at this optimal length for the remainder of the experiment.

Fura-2 loading

Muscle strips were incubated in PSS (22°C) containing 5 μM of the acetoxymethyl ester of fura-2 (fura-2/AM) aerated with 94% O_2 and 6% CO_2 for 3 h (Ozaki *et al*., 1990; Jones *et al*., 1994). The fura-2/AM was dissolved in dimethylsulphoxide

(DMSO) and 0.02% cremophor. After fura-2 loading, the strips were washed with normal PSS (37°C) for 30–50 min to remove extracellular fura-2/AM and DMSO, and to allow de-esterification of any remaining cytosolic fura-2/AM. All further manoeuvres were performed at 37°C.

Fura-2 fluorescence measurements

Fura-2 fluorescence intensity was measured by a photometric system (Scientific Instruments, Heidelberg, Germany, model ph2) which measures optical and mechanical parameters of isolated tissue simultaneously. This system previously has been described in detail (Güth & Wojciechowski, 1986). Light from a xenon high-pressure lamp was monochromatically filtered to restrict excitation light to 340 nm, 360 nm and the 380 nm wavelengths. Excitation light at these three wavelengths was alternated every 2 ms and focused by a high-aperture objective onto the muscle strips. Surface fluorescence emitted from the strips was filtered at 500 ± 5 nm and detected by a photomultiplier assembly. Illumination intensity of the excitation light passing through the cuvette was detected by an absorbance monitor. The photomultiplier signal was normalized by this absorbance to minimize the influence of fluctuations in the intensity of the excitation lights. The emission fluorescence intensities due to excitation at 340 nm (F_{340}), 360 nm (F_{360}) and 380 nm (F_{380}) wavelengths were measured and stored on a personal computer. The ratio of intensities at 340 nm and 380 nm excitation (F_{340}/F_{380}) was used as an index of $[Ca^{2+}]_i$ (Pabelick *et al*., 1997).

Measurement of Mn^{2+} influx

The quenching of fura-2 fluorescence by Mn^{2+} has been used to estimate Ca^{2+} influx in smooth muscle and other cell types (Jacob, 1990; Mertz *et al*., 1990; Chen & Rembold, 1992; Chen & van Breemen, 1993). Mn^{2+} rapidly binds to fura-2 and quenches its fluorescence. Mn^{2+} can be admitted to the cell via calcium channels. The rate of calcium influx can be estimated by the rate of Mn^{2+} influx (Jacob, 1990; Mertz *et al*., 1990; Chen & Rembold, 1992; Chen & van Breemen, 1993) under the following conditions. When Mn^{2+} is added to the extracellular fluid bathing muscle cells loaded with fura-2, a decline in fluorescence is observed. The rate of decline in fura-2 fluorescence, as measured by F_{360} (the isobestic wavelength for fura-2), is an index of Mn^{2+} and hence, calcium influx (Jacob, 1990; Mertz *et al*., 1990; Chen & Rembold, 1992; Chen & van Breemen, 1993). We have previously validated this technique in airway smooth muscle (Pabelick *et al*., 1997).

To measure Mn^{2+} influx, 0.1 mM $MnCl_2$ was added to the perfusate solutions and fluorescence signals were measured over the next 10 min. The tissue was then lysed by the addition of water plus 0.5 mM $MnCl_2$ and background fluorescence was measured. The F_{360} signal was normalized by considering the signal one minute before the addition of Mn^{2+} to be 1.0 and the background fluorescence after lysis to be 0. The Mn^{2+} influx rate was calculated by measuring the slope of the normalized F_{360} signal between 15 and 45 s after the onset of quenching, a period when the signal was most linear (Mertz *et al*., 1990; Pabelick *et al*., 1997).

Materials

Ketamine enantiomers were kindly provided by Parke-Davis (Ann Arbor, MI). All other drugs and chemicals were purchased from Sigma Chemical Company (St. Louis, Missouri). Stock solutions of fura-2/AM were prepared in dimethylsulphoxide (DMSO); all other solutions and drugs were prepared in distilled water.

To confirm the identity of the enantiomers, samples were analysed by gas chromatography/mass spectrometry (Baselt, 1979). The commercially available racemate was used as a calibrating standard to calculate the relative concentration of ketamine in the two other samples. The ketamine concentration in the **R**(−)-ketamine sample was 101% and in the **S**(+)-ketamine sample 99% (relative to the racemate).

Enantiomers can form spontaneously racemates in aqueous solution. To determine the rate of racemization of one form of the enantiomers into the other, aliquots of PSS containing 100 μM of one form of the enantiomers were examined for the presence of the other form by liquid chromatography on a Chira AGP column with u.v.-detection at 210 nm. The mobile phase consisted of 2.5% (v/v) 2-propranolol in phosphate buffer (pH = 7, $\mu = 0.02$) and the quantitation limit was 1% (the analyses were performed by Dr M. Bielenstein, Apoteksbolaget, Stockholm). No racimization was detected in either enantiomer solution.

Statistics

Multiple comparisons were performed by analysis of variance (ANOVA). Single comparisons were made by paired or unpaired *t* tests. A $P \leqslant 0.05$ was considered to be significant. In all studies, *n* refers to the number of dogs studied. Values are presented as mean ± s.d.

Results

Effects of ketamine enantiomers on force and F_{340}/F_{380} in strips stimulated with ACh

After contractions induced by 0.1 μM ACh (EC_{50}) had stabilized (10 min), the strips were perfused with increasing concentrations of each enantiomer and the racemate (1–100 μM), which produced concentration-dependent decreases in both force and F_{340}/F_{380} (Figures 1 and 2). The effect of **R**(−)-ketamine on both force and F_{340}/F_{380} was significantly greater than that produced by **S**(+)-ketamine ($P < 0.0001$ for force and $P < 0.0005$ for F_{340}/F_{380}, $n = 6$, ANOVA); the effect of the racemate was intermediate. The difference between the effects of the enantiomers was most pronounced at 100 μM; **R**(−)-ketamine relaxed the strips to 32% of initial force, whereas **S**(+)-ketamine relaxed the strips to only 90% of initial force. The effects of the racemate and the enantiomers on both force and F_{340}/F_{380} were reversible after washout (data not shown).

*Effect of **R**(−)- and **S**(+)-ketamine on Mn^{2+} influx*

R(−)- or **S**(+)-ketamine (100 μM) was added to the strips 10 min after contraction with 0.1 μM ACh. For each experi-

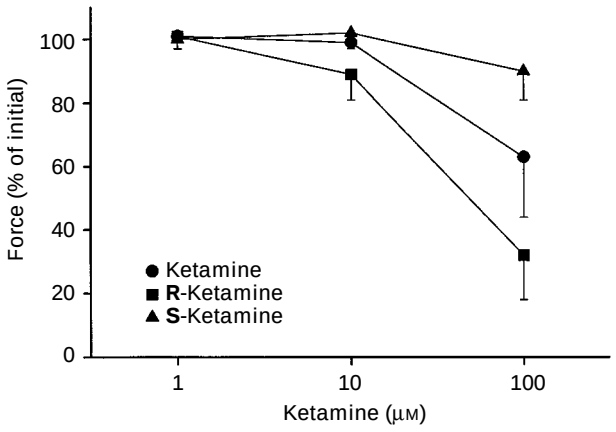

Figure 1 Effect of **S**(+)-ketamine, **R**(−)-ketamine and racemate (1 μM–100 μM) on isometric force in canine smooth muscle strips contracted with 0.1 μM ACh ($n = 6$). Both the racemate and the enantiomers caused a dose-dependent reduction in isometric force. The effect of **R**(−)-ketamine was significantly greater than that of **S**(+)-ketamine, with the racemate intermediate. Values are mean and vertical lines show s.d.

ment, another strip from the same dog did not receive ketamine and served as a control.

Figure 3 shows the results of Mn^{2+}-induced quenching of fura-2 performed 20 min after the beginning of the contraction with 0.1 μM ACh. In control strips, the addition of 0.1 mM $MnCl_2$ produced a rapid decrease in F_{360}, indicating that Mn^{2+} entered the cell and quenched fura-2. Ten minutes after the addition of Mn^{2+}, the strips were lysed with water plus 0.5 mM $MnCl_2$, producing a further decrease in F_{360} to the level of background fluorescence. The addition of 100 μM **R**(−)-ketamine at 10 min after the beginning of the contraction (i.e., 10 min before Mn^{2+}-induced quenching), decreased the rate of decline of F_{360} from 0.15 ± 0.04 min^{-1} (control) to 0.08 ± 0.02 min^{-1} ($P < 0.0005$, paired t test, a 45% decrease in influx rate, $n = 6$). The addition of 100 μM **S**(+)-ketamine in other strips at the same time after the beginning of the contraction also caused a significant decrease in the rate of decline of F_{360} (from 0.16 ± 0.03 min^{-1} (control) to 0.09 ± 0.01 min^{-1}, $P < 0.001$, paired t test, a 44% decrease in influx rate, $n = 6$). Thus, both enantiomers inhibited the rate of Mn^{2+} influx (and hence, calcium influx) during ACh-mediated contractions (Figure 3) but the enantiomers did not significantly differ in their ability to decrease Mn^{2+} influx ($P = 0.782$, unpaired t test).

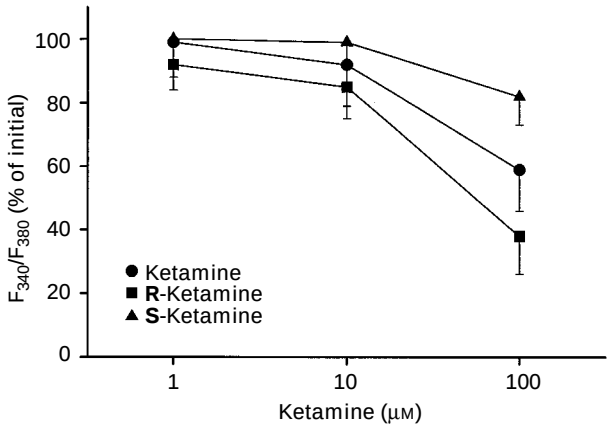

Figure 2 Effect of **S**(+)-ketamine, **R**(−)-ketamine and racemate (1 μM–100 μM) on ratio of fluorescence intensities due to excitation at 340 and 380 nm (F_{340}/F_{380}, an index of intracellular calcium concentration) in canine tracheal smooth muscle strips contracted with 0.1 μM ACh ($n = 6$). Both the racemate and the enantiomers caused a significant dose-dependent decrease in F_{340}/F_{380}. The effect of **R**(−)-ketamine was significantly greater than that of **S**(+)-ketamine, with the racemate intermediate. Values are mean and vertical lines show s.d.

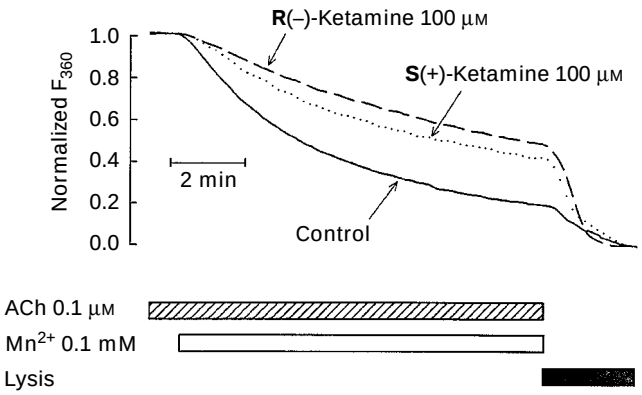

Figure 3 Fluorescence intensity due to excitation at 360 nm (F_{360}) in three strips of canine tracheal smooth muscle during stimulation with 0.1 μM ACh. Both **R**(−)-ketamine (100 μM) and **S**(+)-ketamine (100 μM) decreased the rate of decline of F_{360} produced by the addition of 0.1 mM Mn^{2+}. F_{360} was normalized to values before Mn^{2+} addition and after lysis.

Effects of ketamine enantiomers on force and F_{340}/F_{380} in strips stimulated with isotonic KCL

After contractions induced by 24 mM isotonic KCl (a concentration equal approximately EC_{50} for KCl) had stabilized (10 min), the strips were perfused with increasing concentrations of the enantiomers and the racemate (1–100 μM) Figures 4 and 5). Both enantiomers produced a small but statistically significant decrease in both force and F_{340}/F_{380} ($P < 0.0004$ for force and $P < 0.0001$ for F_{340}/F_{380}, ANOVA). With *post-hoc* tests, this decrease was significant only for 100 μM ketamine. There was no significant difference between **S**(+)-ketamine, **R**(−)-ketamine, and the racemate in their effects on either force ($P = 0.425$) or F_{340}/F_{380} ($P = 0.604$).

Effects of ketamine enantiomers on force and F_{340}/F_{380} in human airway strips stimulated with ACh

Additional experiments were performed to determine if stereospecific effects of ketamine enantiomers are also present in human airway. Strips of human bronchial smooth muscle obtained from surgical specimens were contracted at optimal length with 1 μM ACh (EC_{50}). Like the canine tracheal smooth muscle strips there was a consistent difference in the amount of relaxation caused by **R**(−)- and **S**(+)-ketamine. **R**(−)-ketamine relaxed the strips to 48 ± 3% of initial force, whereas **S**(+)-ketamine relaxed the strips to only 81 ± 13% of initial force ($n = 2$). This finding suggests that under *in vitro* conditions differential effects of ketamine enantiomers are also present in human airways.

Discussion

The ability of ketamine enantiomers to relax canine ASM differed during muscarinic receptor stimulation but not during contractions produced by isotonic KCl. The differential effects during ACh-induced contractions could not be attributed to a difference in calcium influx measured with a Mn^{2+} quenching technique.

Stereospecific effects of ketamine enantiomers were first noted in the central nervous system (CNS). In animals and man, the two enantiomers differ in their analgesic (Ryder *et al.*, 1978) and anaesthetic (White *et al.*, 1980; 1985) potencies, with **S**(+)-ketamine being about 2–4 times more potent than

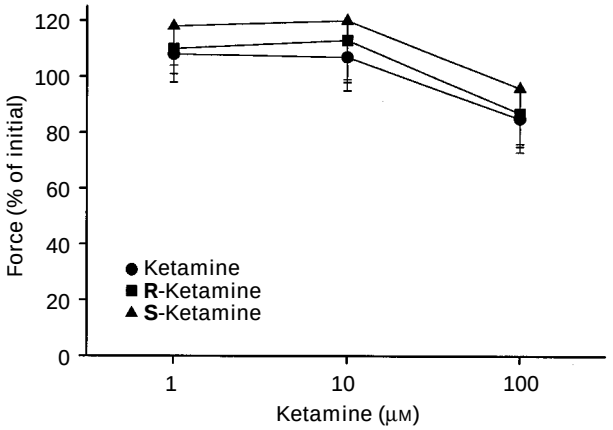

Figure 4 Effect of **S**(+)-ketamine, **R**(−)-ketamine and racemate on canine smooth muscle strips contracted with 24 mM KCl (EC_{50}, $n = 6$). There was no significant difference between the **R**(−)- and **S**(+)-ketamine in their effects on isometric force. Values are mean and vertical lines show s.d.

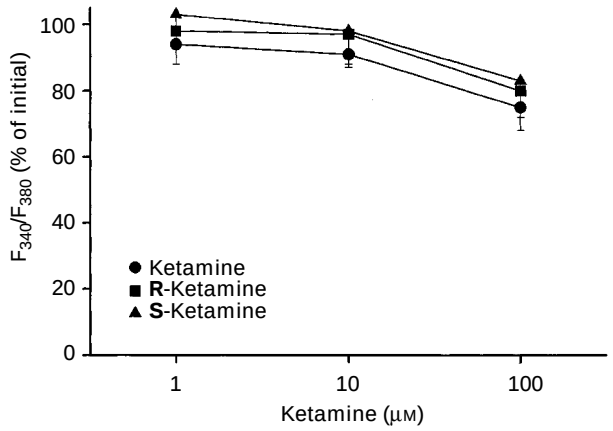

Figure 5 Effect of **S**(+)-ketamine, **R**(−)-ketamine and racemate on ratio of fluorescence intensities due to excitation at 340 and 380 nm (F_{340}/F_{380}), as an index of intracellular calcium concentration) in canine tracheal smooth muscle strips contracted with 24 mM KCl (EC_{50}, $n=6$). There was no significant difference between the **R**(−)- and **S**(+)-ketamine in their effects on isometric force. Values are mean and vertical lines show s.d.

R(−)-ketamine. Ketamine analgesia results from non-competitive blockade at the phencyclidine (PCP) site of the N-methyl-D-aspartate (NMDA) receptor, a ligand-gated ion channel (Klepstad *et al*., 1990). The affinity of ketamine enantiomers to the PCP binding site within the NMDA receptor correlates well with their analgesic potency (Klepstad *et al*., 1990). This finding that the analgesic effect of ketamine is stereoselective is consistent with a receptor-mediated pharmacological mechanism, suggesting that differences in binding affinity with other targets may be present in other tissues (Klepstad *et al*., 1990).

Studies in non-neural tissues showed that there is a differential effect of ketamine enantiomers on catecholamine uptake mechanisms (Lundy *et al*., 1986) and a stereospecific effect on heart rate, isovolumetric systolic left ventricular pressure and oxygen consumption of guinea-pig isolated perfused hearts (Graf *et al*., 1995). However, other studies revealed equipotent cardiodepressant effects in myocytes (Sekino *et al*., 1996) and no stereospecific spasmolytic difference in guinea-pig tracheal smooth muscle contracted with histamine (Hirota *et al*., 1996). The discordance between Hirota's findings and our results could be due to species differences or the agonist used for inducing ASM contraction. However, Hirota *et al*. did find stereospecific differences between the enantiomers in their ability to potentiate the relaxing effect of adrenaline, with the **S**(+) enantiomer being more potent.

In a previous study we showed that ketamine relaxes canine tracheal smooth muscle (CTSM) contracted with the physiological agonist ACh by decreasing $[Ca^{2+}]_i$. This decrease in $[Ca^{2+}]_i$ was caused by an inhibition of calcium influx measured by a Mn^{2+} quenching technique (Pabelick *et al*., 1997). By use of similar protocols for the enantiomers a significant difference between **R**(−)- and **S**(+)-ketamine in their effects on force and $[Ca^{2+}]_i$ was found during ACh stimulation. In contrast, the enantiomers did not differ in their ability to inhibit calcium influx. Further, in CTSM contracted with isotonic KCl there was no difference between **S**(+)-ketamine and **R**(−)-ketamine in their relatively modest effects on force and $[Ca^{2+}]_i$. We propose the following explanation of these results.

Two types of channels can mediate calcium influx during ASM contraction (Torphy & Hay, 1990). These are denoted as voltage-operated calcium channels (VOCCs) and receptor-operated calcium channels (ROCCs). The fact that the enantiomers differed in their ability to decrease $[Ca^{2+}]_i$, but not Mn^{2+} influx, suggests that the Mn^{2+} influx technique may not measure total Ca^{2+} influx. Previous results have suggested that Mn^{2+} is admitted into ASM cells via VOCCs (Murray & Kotlikoff, 1991; Pabelick *et al*., 1997), but that Mn^{2+} may not transverse ROCCs (Murray *et al*., 1991; 1993). According to this interpretation, the finding that the enantiomers equally depressed Mn^{2+} influx implies that they do not differentially affect the function of VOCCs. This result is supported by the equal effects of the enantiomers on isotonic KCl-induced contractions, in which calcium influx and the increase in $[Ca^{2+}]_i$ are maintained primarily by VOCCs. A whole-cell patch-clamp study of porcine tracheal smooth muscle cells (Yamakage *et al*., 1995) also showed that ketamine (racemate) in clinically used concentrations (100 μM) had a modest effect on the whole-cell inward Ca^{2+} current through VOCCs. Rather, the differential effect of the enantiomers on force and $[Ca^{2+}]_i$ appears to be caused by a differential action on system(s) regulating ROCCs. The mechanism of this action is unknown. There is no evidence that NMDA receptors exist in ASM. However, the stereospecific action implies a specific binding or other specific association with a regulatory site. Durieux (1995) showed that ketamine depressed signalling pathways mediated by rat M_1 muscarinic receptors in *Xenopus* oocytes, and similar effects may be present in canine ASM. Further investigation of the role of ROCCs is limited by the lack of specific pharmacological antagonists (Merritt *et al*., 1990; Leung *et al*., 1996).

Although comparisons between *in vitro* and *in vivo* drug concentrations across species may be of limited value, peak plasma concentrations in man have been shown to be approximately 60 μM for an intravenous dose of 2 mg kg^{-1} ketamine (Idvall *et al*., 1979), indicating that the range of ketamine concentrations studied may have clinical relevance. These results suggest that the specific administration of **R**(−)-ketamine to treat bronchospasm may be advantageous. Since **S**(+)-ketamine is the more potent anaesthetic, **R**(−)-ketamine may be preferable in the treatment of bronchospasm because bronchodilating properties are maximized and CNS effects may be minimized. However, further studies are required to confirm differential effects of ketamine enantiomers *in vivo* before this approach can be recommended.

In summary, the **R**(−)-enantiomer is a more potent bronchodilator than the **S**(+)-enantiomer of ketamine during ACh-mediated ASM contraction. This effect appears to be caused by differential actions on receptor-operated calcium channels.

The **S**(+)- and **R**(−)-enantiomers of ketamine were kindly supplied by Parke-Davis (Ann Arbor, Michigan).

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