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INCREASE IN CALCIUM SENSITIVITY OF CARDIAC MYOFIBRILS CONTRIBUTES TO THE CARDIOTONIC ACTION OF SULMAZOLE

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Abstract—The aim of this study was to investigate whether increasing calcium sensitivity of myofibrils plays a role in the positive inotropic activity of the cardiotonic agent sulmazole. We studied the effects of the stereoisomers of sulmazole on cardiac contractility *in vivo* and *in vitro*, arterial blood pressure, cardiac (Na–K)ATPase activity, cAMP/cGMP-phosphodiesterase activity of cardiac and smooth muscle tissue and calcium sensitivity of skinned myocardial fibres. Both stereoisomers of sulmazole were equipotent vasodilators *in vivo* and this can be explained by their equipotent cAMP- and cGMP-phosphodiesterase inhibitory activities in smooth muscle tissue. However, (+)sulmazole was a much stronger positive inotropic agent than (−)sulmazole *in vivo* and *in vitro*. This difference in inotropic activity cannot be explained by cAMP- or cGMP-phosphodiesterase inhibition or (Na–K)ATPase inhibition in cardiac tissue. Only (+)sulmazole produced a dose-dependent increase in calcium sensitivity of skinned myocardial fibres. Therefore, the calcium sensitizing effect on myofibrils evoked by (+)sulmazole might be responsible for the difference in inotropic activity observed between the stereoisomers of sulmazole.

Positive inotropic drugs are used in the treatment of congestive heart failure. The drugs used are structurally related to cardiac glycosides or catecholamines. However, a number of substances chemically unrelated to digitalis or catecholamines have recently been developed which also evoke positive inotropic effects [1–5]. For some of these substances new mechanisms of action have been proposed which may contribute to the positive inotropic activity. An example of such a new mechanism of action is the increase in the sensitivity towards calcium of myofibrils [6]. Sulmazole (= AR-L 115 BS) was the first clinically investigated cardiotonic agent for which a calcium sensitizing action in chemically skinned myocardial fibres has been described [7–9]. However, additional mechanisms of action have been proposed for sulmazole: cAMP-phosphodiesterase inhibition [2, 10–13], inhibition of (Na-K)ATPase [2, 14], release of calcium from sarcoplasmic reticulum and inhibition of calcium reuptake [15], displacement of calcium from non-specific ligand sites within the protein matrix of the cytosol [16].

We investigated some of these effects with the stereoisomers of sulmazole (Fig. 1) to find out whether an increase in the sensitivity to calcium of the myofibrils contributes to the positive inotropic effects induced by sulmazole *in vivo* and *in vitro*.

(±) SULMAZOLE

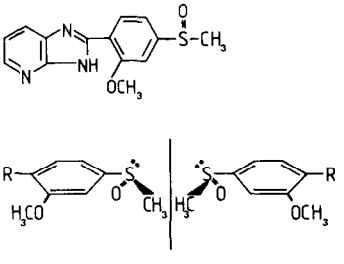

Fig. 1. Structural formula of sulmazole and the position of the asymmetry in the molecule.

MATERIAL AND METHODS

Inhibition of high affinity cAMP- and cGMP-phosphodiesterase in vitro. The inhibition of high affinity cAMP- or cGMP-phosphodiesterase in crude enzyme preparations of different tissues was determined by the modified radioisotope method described by Poech [17]: 1 μmol/l ^{3}H-cAMP (0.1 μmol/l ^{3}H-cGMP) was added as substrate and after incubation the radiolabelled hydrolysis product ^{3}H-AMP (^{3}H-GMP) was quantitatively removed by $ZnSO_4$ and $Ba(OH)_2$. Unlabelled AMP (GMP) was added to the buffer to prevent any further breakdown

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of the enzymatic product ^{3}H-AMP (^{3}H-GMP) to ^{3}H-adenosine (^{3}H-guanosine).

The different tissues were homogenized in a Potter–Elvehjem with 4 volumes of 0.05 mol/l Tris–HCl (pH = 7.4) and centrifuged in a Heraeus–Christ Varifuge ST (rotor 5220) at 6,500 *g* for 15 min at 4°. The supernatant was frozen in portions at −30°. Before assay, the supernatant was diluted so that a 100 µl in the volume of sample in the assay system produced 20–30% hydrolysis of substrate within the incubation time.

The reaction mixture—consisting of 50 µl of 0.3 mol/l Tris–HCl (pH = 7.4) (containing 9 mmol/l $MgCl_2$, 3 mmol/l AMP, 3 µmol/l cAMP and 15 µl ^{3}H-cAMP (NET-275, New England Nuclear) per 20 ml), 50 µl of test compound dissolved in water or 50 µl of water in control experiments and 50 µl of crude enzyme dilution—was incubated for 15 min at 37° in 1.5 ml Eppendorf microtubes.

The reaction was stopped by 250 µl $ZnSO_4$ (0.266 mol/l). After addition of 250 µl of $Ba(OH)_2$ (0.266 mol/l) and mixing, the samples were centrifuged in a Heraeus Biofuge A centrifuge at 12,000 *g* for 2 min. 500 µl of the supernatant was mixed with 4.5 ml of Instagel in a minivial and the ^{3}H-activity which responds to residual substrate was determined in a Packard 460 C liquid scintillation counter. All incubations were performed in triplicate.

The same procedure as described above was used for the inhibition of cGMP-phosphodiesterase assays. However, the reaction mixture contained 0.3 mol/l Tris–buffer (pH = 7.4) with 9 mmol/l $MgCl_2$, 3 mmol/l GMP, 0.1 µmol/l cGMP and 15 µl ^{3}H-cGMP (NET-337, New England Nuclear) per 20 ml.

We did not always obtain 50% inhibition of the enzymes even at 1 mmol/l, because some stereoisomers of sulmazole were not always very effective inhibitors of the phosphodiesterase. For this reason we calculated $IC_{35\%}$-values for three separate measurements with corresponding 95% confidence intervals. The $IC_{35\%}$-values represent the concentration of the test compound in µmol/l which inhibited the enzymatic hydrolysis of ^{3}H-cAMP or ^{3}H-cGMP with respect to control by 35%. The $IC_{35\%}$-values were calculated from the dose–response curves by linear regression analysis on a Wang 2200 computer system.

Inhibition of cat heart (Na–K)*ATPase activity.* Cat heart cell membrane preparations were obtained using the partial purification procedure described previously [18]. The cat heart membrane preparation included the NaI treatment and was homogenized in 1 mmol/l EDTA (pH = 7.25; 60 ml). The (Na–K)ATPase activity, determined by the coupled optical assay method [19], was 0.25 µmol ATP hydrolysed/mg protein/min for cat heart preparations. Protein was measured by the method of Lowry *et al.* [20] using bovine serum albumin as the standard protein. Inhibition of (Na–K)ATPase by 1 mmol/l ouabain was 85% of total activity. K_d-values were calculated by Scatchard analysis.

The heart cell membranes were incubated in a solution containing 3 mmol/l $MgCl_2$, 3 mmol/l PO_4^{2-}, 50 mmol/l imidazole–HCl (pH = 7.25), (±)sulmazole, (+)sulmazole or (−)sulmazole 0, 10, 100 or 1000 µmol/l, final volume 1 ml, at 37° for 30 min. The (Na–K)ATPase activity of 0.8 ml of this suspension was measured at 366 nm by the coupled optical assay method. Unspecific (Na–K)ATPase activity ((Na–K)ATPase activity in the presence of 1 mmol/l ouabain) was subtracted for all drug concentrations.

^{3}H-*ouabain binding to cat heart cell membranes.* The procedure used for these experiments have been described elsewhere [18]. A rapid filtration method was used to separate free ouabain from membrane bound ouabain. Nonspecific binding (binding in the presence of 1 mmol/l unlabelled ouabain) was less than 5% of total radioactivity bound to the cell membranes and was subtracted. All experiments were performed in triplicate. The heart cell membranes were incubated in a solution containing 3 mmol/l $MgCl_2$, 3 mmol/l PO_4^{2-}, 50 mmol/l imidazole–HCl (pH = 7.25) about 2 nmol/l ^{3}H-ouabain, test substance 0–1 mmol/l, in a final volume of 2.0 ml at 37° for 3 hr. ^{3}H-Ouabain (specific activity 20.9 Ci/mmol) was purchased from New England Nuclear.

Experiments on skinned cardiac muscle fibres. The experiments on skinned cardiac muscle fibres were performed according to the method as described by Rüegg *et al.* [21]. Subendocardial muscle fibres (about 4 mm in length and 0.1 mm in diameter) were prepared from porcine trabecula septomarginalis and extracted for 12 hr in a solution containing 50% glycerol and 50% of a buffer containing 20 mmol/l imidazole, 10 mmol/l sodium azide, 2 mmol/l dithioerythritol and 0.5% Lubrol WX, pH = 7.0 at 4°. The preparations were subsequently stored in the same solution but without Lubrol at −20° for several days as described by Herzig *et al.* [7].

After mounting the preparations with fast setting glue on an isometric force transducer (type AE 801 AME, Horten, Norway) and a glass rod attached to a micromanipulator, the fibres were relaxed by immersion in a solution containing 10 mmol/l ATP, 12.5 mmol/l $MgCl_2$, 5 mmol/l EGTA, 25 U/ml creatine phosphate kinase, 20 mmol/l imidazole, 5 mmol/l NaN_3 and 10 mmol/l creatine phosphate resulting in a free calcium concentration of <10 nmol/l. Contraction was induced by immersion in a similar solution in which EGTA was replaced with EGTA–calcium buffer. The buffered calcium concentration was determined from the ratio of EGTA to calcium–EGTA according to Portzehl *et al.* [22] using an apparent dissociation constant of 1.6 µmol/l for the calcium–EGTA buffer at pH = 6.7 at 20°. Free calcium concentrations were calculated as described by Fabiato and Fabiato [23].

Experiments on isolated papillary muscles of guinea-pigs. Guinea-pigs of either sex (Dunkin–Hartley–Pirbright, 300–400 g) were killed by cervical dislocation. Right ventricular papillary muscles (diameter 0.5–1.0 mm) were rapidly dissected from the isolated heart and mounted in an organ bath (volume 70 ml). The muscles were stimulated by rectangular voltage pulses (2 msec in duration) about 10% above threshold with two platinum electrodes close to the base of the muscle. Force was recorded isometrically by an inductive force transducer connected to an 8-channel recorder. The resting force

was kept constant at 5 mN throughout the experiment. An equilibrium period of at least 1 hr at a frequency of stimulation of 1 Hz preceded each experiment. The bath solution was a modified Krebs–Henseleit solution of the following composition: NaCl 114.9 mmol/l, $NaHCO_3$ 24.88 mmol/l, KCl 4.69 mmol/l, KH_2PO_4 1.18 mmol/l, $CaCl_2$ 2.7 mmol/l, $MgSO_4$ 0.78 mmol/l, glucose 10 mmol/l. Temperature was maintained at 37°. The solution was continuously gassed with a mixture of $O_2 + CO_2$ (95 + 5 vol%). The pH of the solution was between 7.40 and 7.50. Cumulative dose–response curves were constructed at a constant frequency of stimulation of 1 Hz.

Experiments on pithed guinea-pigs. Positive inotropic and hypotensive effects of the stereoisomers of sulmazole were investigated in pithed guinea pigs as described previously [24]. Male guinea pigs (Dunkin–Hartley–Pirbright, 400–500 g) were anaesthetized with hexobarbitone–sodium (150 mg/kg, i.p.). The trachea was cannulated and the animals were pithed by inserting a steel rod (2.0 mm in diameter) through the skull down through the spinal cord. Immediately after pithing, the trachea cannula was connected with a positive pressure pump to allow artificial respiration. The animals were ventilated with a mixture of 50% O_2 + 50% N_2 (400 ml/min) at a frequency of 40 cycles per minute. The right jugular vein was cannulated for intravenous administration of drugs. One common carotid artery was used for the registration of arterial blood pressure (Bell and Howell pressure transducer, 4-327-I). A Millar tip pressure transducer (Millar PR-249) was introduced into the left ventricle via the other carotid artery. Left ventricular pressure (LVP), LV–dP/d$t$max and heart rate triggered by the ventricular pressure signals were continuously recorded. Recordings of LVP, LV-dP/d$t$max, arterial blood pressure and heart rate were displayed on an 8-channel recorder. Body temperature was maintained at approximately 37° using a heating pad. Substances were administered in a volume of 0.5 ml/kg as single bolus injections. Five to six doses of a compound were given to each animal. Care was taken to ensure a return to pre-injection values between subsequent doses.

The hypotensive effects of the stereoisomers of sulmazole were measured in a separate series of experiments. The right common carotid artery of pithed guinea pigs was used for registration of arterial blood pressure. The other carotid artery was used for continuous infusion of noradrenaline. Diastolic blood pressure was increased to about 95 mm Hg by adjusting the infusion rate. The maximal volume of infusion was 0.05 ml/min. After stabilisation of cardiovascular parameters (in general within 5 min) the substances were cumulatively injected into a jugular vein in a volume of 0.5 ml/kg. A cumulative dose–response curve was obtained with respect to decrease in diastolic blood pressure.

Experiments on anaesthetized cats. Cats of either sex (2.5–4.5 kg) were anaesthetized with pentobarbitone-sodium (40 mg/kg i.p. and subsequently 8 mg/kg/hr i.v.). The trachea was cannulated and the animals were allowed to breathe spontaneously. The right common carotid artery was cannulated for insertion of a Millar tip pressure transducer (PC 350A) into the left ventricle. Left ventricular pressure, heart rate triggered from the left ventricular pressure pulse signals and LV-dP/d$t$max were registered continuously. Arterial blood pressure was measured by a pressure transducer (Bell and Howell, 4-327-I) inserted in the abdominal aorta via the right femoral artery. Arterial blood pressure, heart rate and LV-dP/d$t$max were recorded on an 8-channel recorder. Intravenous administration of drugs was performed via the right femoral vein. The drugs were given in a volume of 0.1 ml/kg.

Drugs and chemicals used. All chemical used were of analytical grade and were obtained from Boehringer Mannheim (Mannheim, F.R.G.), E. Merck (Darmstadt, F.R.G.), Serva Biochemicals (Heidelberg, F.R.G.) and Sigma (Muenchen, F.R.G.). Hexobarbitone–sodium (Evipan) and pentobarbitone–sodium (Nembutal) were purchased from Bayer (Leverkusen, F.R.G.) and Seva (Bad Segeberg, F.R.G.), respectively. The (+) and (−) enantiomers of sulmazole were provided by Dr Landgraf of the Galenical Department of Thomae, Biberach. The purity of the isomers was higher than 99.5%. The compounds were dissolved in 0.9% NaCl solution or water.

Statistics. Experimental values are given as mean ± SEM. The $IC_{35\%}$ values for phosphodiesterase inhibition are presented as mean values with corresponding 95% confidence intervals. The statistical significance of differences between mean values was estimated by Student's t-test (paired when applicable). A significance level of $P = 0.05$ was used throughout.

RESULTS

cAMP- and cGMP-phosphodiesterase inhibition

Because inhibition of cyclic nucleotide phosphodiesterase affect a variety of physiological responses like cardiac contractility, vascular relaxation and platelet aggregation, we investigated the cAMP- and cGMP-phosphodiesterase inhibitory effects of the stereoisomers of sulmazole in different preparations. The results are summarized in Table 1. It was found that (+) and (−) sulmazole were equipotent phosphodiesterase inhibitors in the aorta preparations of two different species. The results with human thrombocytes indicate that there was no difference in potency of cAMP-phosphodiesterase or of cGMP-phosphodiesterase inhibition between the stereoisomers of sulmazole. The experiments on crude heart preparations of guinea-pig and swine showed that the stereoisomers were equipotent inhibitors of the cardiac phosphodiesterases.

This was also demonstrated by comparing the phosphodiesterase inhibitory activities of the stereoisomers of sulmazole in porcine and guinea pig heart preparations at 0.1 mmol/l. The extent of inhibition (%) as mean value with 95% confidence interval produced by 0.1 mmol/l of (+) and (−) sulmazole on cAMP- and cGMP-phosphodiesterases is presented in Table 2.

Table 1. Phosphodiesterase inhibitory activity of (+) and (−)sulmazole

	cAMP-phosphodiesterase $IC_{35\%}$ μmol/l		cGMP-phosphodiesterase $IC_{35\%}$ μmol/l	
	(+)isomer	(−)isomer	(+)isomer	(−)isomer
Bovine aorta	60 (42–79)	78 (46–130)	11 (9–14)	10 (8–13)
Swine aorta	22 (8–58)	137 (75–249)	17 (12–23)	15 (12–18)
Human thrombocyte	23 (15–35)	32 (26–38)	5 (4–6)	2 (1–4)
Swine heart	123 (80–188)	216(169–276)	77 (53–112)	130 (86–196)
Guinea-pig heart	479(387–593)	277(236–326)	740(540–1015)	432(349–535)

Data represent mean values with 95% confidence intervals within parentheses.

(Na–K)ATPase inhibition and ^{3}H-ouabain binding experiments

The concentration of ouabain which inhibited (Na–K)ATPase activity by 50% was 5.8 nmol/l for cat heart preparations (Fig. 2). (−)Sulmazole showed a weak (<10%) inhibition of (Na–K)ATPase at 1 mmol/l. However, (+)sulmazole produced a more pronounced inhibition of (Na–K)ATPase activity (Fig. 2). The IC_{50}-value of (+)sulmazole for (Na–K)ATPase inhibition was about 0.85 mmol/l. A slight (<20%) but significant inhibition of (Na–K)ATPase was observed at 0.1 mmol/l of (+)sulmazole whereas no effects were measured at 10 μmol/l with the (+)isomer.

Ouabain bound specifically to cat ($K_d = 2.6 \times 10 - 9$) cardiac cell membrane preparations. (−)Sulmazole (1 mmol/l) was relatively ineffective at displacing ^{3}H-ouabain from its specific binding sites (Fig. 3). On the other hand, (+)sulmazole showed a more pronounced inhibitory effect of ^{3}H-ouabain binding (Fig. 3); a significant displacement was observed at 0.1 mmol/l of (+)sulmazole. A concentration of 1 mmol/l of (+)sulmazole reduced ^{3}H-ouabain to 59%.

Skinned myocardial fibers

The detergent-treated preparations developed maximal isometric tension at 14 μmol/l Ca^{2+}. This increase in tension represents 100% tension development. The preparations were subsequently allowed to relax in a solution containing <10 nmol/l Ca^{2+}. Afterwards the preparations were immersed in a solution with a fixed calcium concentration and tension development was observed. After a plateau was reached, this solution was replaced with a similar solution which contained 0.1 mmol/l of either isomer or sulmazole. The *additional* increase in tension observed in the presence of the drug was expressed as a percentage increase in tension. We saw that only (+)sulmazole induced a significant additional increase in tension. This effect was reversible and especially very pronounced at lower calcium concentrations (Fig. 4). However, (−)sulmazole (up to 1 mmol/l) was almost ineffective at increasing the calcium sensitivity of the myofibrils.

The differences in effects on calcium sensitivity induced by (+) and (−)sulmazole were significant ($P < 0.05$). The maximal tension development observed at 14 μmol/l Ca^{2+} was not influenced by (+) or (−)sulmazole. The increase in calcium sensitivity of the myofibrils induced by (+)sulmazole proved to be dose-dependent. Figure 5 shows the dose-related additional increase in tension developed at a fixed calcium concentration of pCa = 6.18.

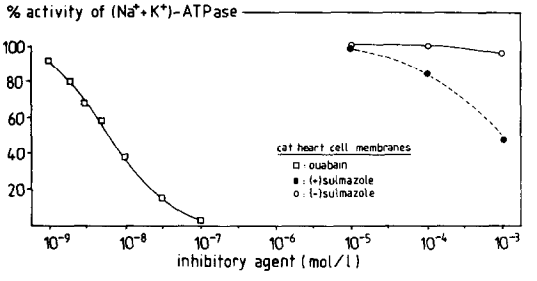

Fig. 2. Inhibition of cat heart (Na–K)ATPase activity by ouabain, (+) and (−)sulmazole. See text for explanation.

Table 2. Phosphodiesterase inhibitory activities of 0.1 mmol/l of (+) and (−)sulmazole in guinea-pig and porcine cardiac preparations

(−)*Sulmazole*:	Guinea-pig heart	cAMP : 22 (17–27) %
		cGMP : 18 (11–25) %
	Porcine heart	cAMP : 22 (15–29) %
		cGMP : 30 (22–38) %
(+)*Sulmazole*:	Guinea-pig heart	cAMP : 15 (10–20) %
		cGMP : 15 (9–21) %
	Porcine heart	cAMP : 32 (21–39) %
		cGMP : 35 (28–42) %

Data represent mean values with 95% confidence intervals within parentheses.

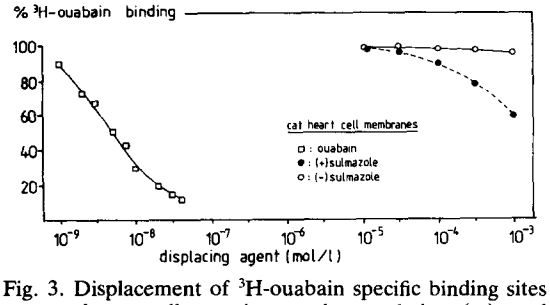

Fig. 3. Displacement of ^{3}H-ouabain specific binding sites to cat heart cell membranes by ouabain, (+) and (−)sulmazole. See text for explanations.

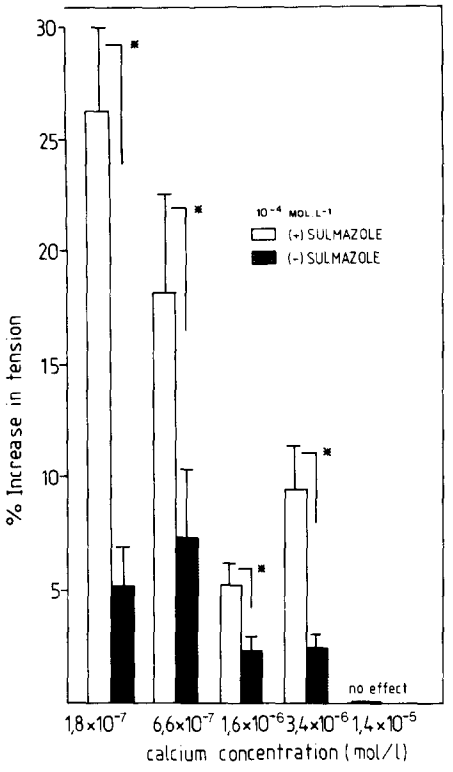

Fig. 4. Increase in force development produced by 0.1 mmol/l of (+) and (−)sulmazole at various free calcium concentrations in chemically skinned porcine myocardial fibres. Data represent mean values ± SEM (N = 7–18). Significant differences in contractile potency between the stereoisomers of sulmazole are indicated by an asterisk ($P < 0.05$).

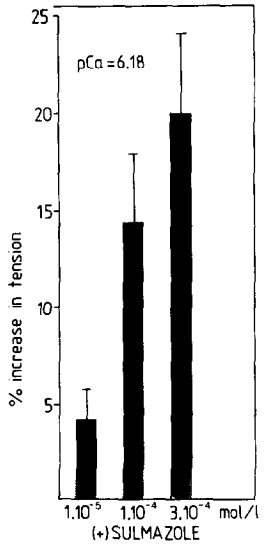

Fig. 5. Increase in force development induced by (+)sulmazole at constant pCa = 6.18 in skinned porcine myocardial fibres. Data represent mean values ± SEM (N = 6).

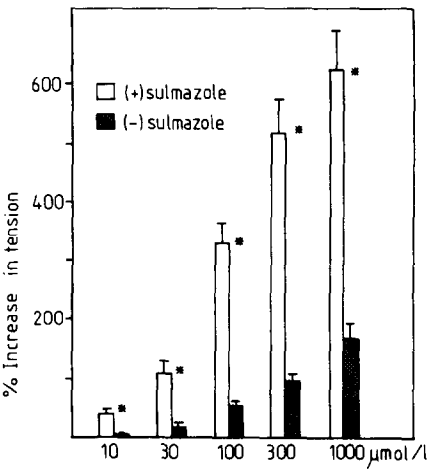

Fig. 6. Increase in force (%) evoked by (+) and (−)sulmazole in isolated guinea-pig papillary muscles; 1 Hz and 2.7 mmol/l Ca^{2+}. Data represent mean values ± SEM (N = 5–6). Significant differences in activity between the stereoisomers are indicated by an asterisk ($P < 0.05$).

Isolated papillary muscles of guinea-pigs

Isometric contraction curves for papillary muscles of guinea pigs were obtained at 1 Hz frequency. (+) and (−)Sulmazole dose-dependently increased the force of contraction of guinea pig papillary muscles. The positive inotropic effects were expressed as a percentage increase of control values. The results are presented in Fig. 6. Resting tension was 1.90 ± 0.33 mN (N = 5) for the (+)sulmazole experiments and 2.96 ± 0.79 mN (N = 6) for the (−)sulmazole experiments. The differences in inotropic activity between (+) and (−)sulmazole were pronounced and statistically significant at all concentrations investigated. The % increase in tension induced by 1000 µmol/l of (+)sulmazole amounted to 627 ± 67% (N = 5) and for 1000 µmol/l of (−)sulmazole to 170 ± 23% (N = 6).

Experiments on pithed guinea pigs

Pithed guinea pigs had a basal value of LV-dP/d$t$max of 1072 ± 61 mm Hg/sec (N = 12). (+)Sulmazole evoked a maximal increase in LV-dP/d$t$max of 1523 ± 115 mm Hg/sec (N = 6), an increase of 136 ± 23% (Fig. 7). The higher dose of (+)sulmazole (100 mg/kg) produced a minor increase in LV-dP/d$t$max, 70 ± 27%, and consequently a bell shaped dose–response curve was obtained. (−)Sulmazole was significantly less active than the (+)isomer. The (−)isomer induced a maximal increase in LV-dP/d$t$max of 492 ± 48 mm Hg/sec (N = 6), an increase of 42 ± 5%. The higher dose of (−)sulmazole (100 mg/kg) increased LV-dP/d$t$max by 29 ± 10%. The control values of diastolic blood pressure during the noradrenaline infusion amounted to 98 ± 3 mm Hg (N = 12) in the pithed guinea pigs. (+)Sulmazole induced a maximal decrease in diastolic blood pressure of 54 ± 5% (Fig. 7). (−)Sulmazole also dose-dependently reduced diastolic blood pressure. The decreases in diastolic blood pressure induced by (−)sulmazole were not significantly different from those evoked by (+)sulmazole.

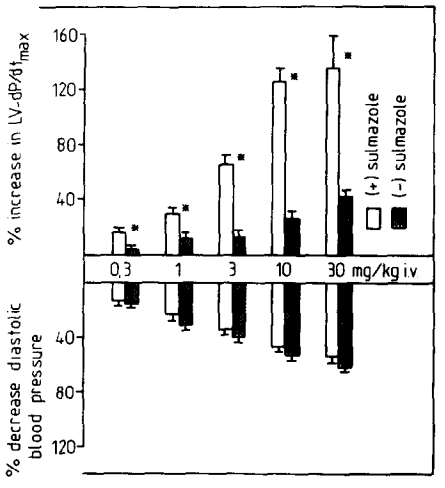

Fig. 7. Cardiovascular effects of (+) and (−)sulmazole in pithed guinea-pigs. Data represent mean values ± S.E.M. (N = 6). Significant differences in activity between the stereoisomers are indicated by an asterisk ($P < 0.05$).

Experiments on anaesthetized cats

In the first series of experiments which examined the effects of (−)sulmazole, pentobarbital-anaesthetized cats had control values of LV-dP/d$t$max and diastolic blood pressure of 4377 ± 251 mm Hg/s (N = 4) and 107 ± 9 mm Hg (N = 4), respectively. (−)Sulmazole dose-dependently increased LV-dP/d$t$max by 8 ± 1 to 13 ± 4% in the dose range 0.1−1 mg/kg i.v. (Fig. 8). Diastolic blood pressure was lowered by (−)sulmazole by 6 ± 2% to 18 ± 2%.

In the second series of experiments, we investigated the effects of (+)sulmazole. The LV-dP/d$t$max and diastolic blood pressure values amounted to 4428 ± 281 mm Hg/sec (N = 4) and 106 ± 8 mm Hg (N = 4) respectively. (+)Sulmazole induced a marked increase in LV-dP/d$t$max, 19 ± 3% to 43 ± 9% (Fig. 8). However, diastolic blood pressure was similarly decreased by 10 ± 2% to 27 ± 4% as compared with (−)sulmazole. The effects on LV-dP/d$t$max induced by (+)sulmazole were significantly higher than the positive inotropic effects evoked by (−)sulmazole. Despite this, the hypotensive effects of (+) and (−)sulmazole were comparable ($P > 0.05$).

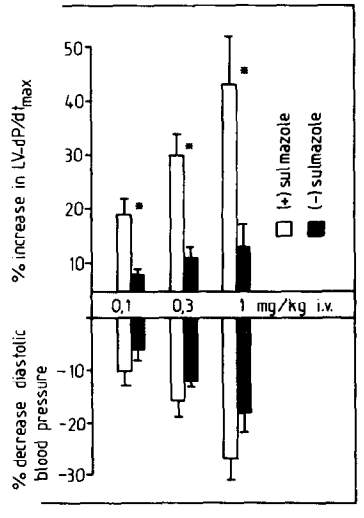

Fig. 8. Cardiovascular effects of (+) and (−)sulmazole in pentobarbitone-anaesthetized cats. Data represent mean values ± SEM (N = 4). Significant differences in activity between the stereoisomers are indicated by an asterisk ($P < 0.05$).

DISCUSSION

The stereoisomers of (±)sulmazole were used in this study to investigate whether an increase in calcium sensitivity of myofibrils might play a role in the positive inotropic effects of sulmazole. We focused our attention on three mechanisms: (1) cAMP- and cGMP-phosphodiesterase inhibition; sulmazole was described to inhibit these enzyme systems [2, 10]. (2) Inhibition of (Na-K)ATPase; Weishaar *et al.* [2] and Honerjaeger *et al.* [14] proposed a role for this system in the inotropic activity of sulmazole. (3) Stimulation of myofibrillar calcium affinity which was reported by Herzig *et al.* [7], Solaro and Rüegg [8] and Blinks and Endoh [9].

Other cellular effects of sulmazole do not probably play a major role in the inotropic activity. Release of calcium from sarcoplasmic reticulum and inhibition of calcium reuptake was described by Trube and Trautwein [15]. However, these effects were only observed at very high concentrations >1 mmol/l of sulmazole. In addition, Weishaar *et al.* [2] found no effects on calcium transport, uptake and release by the sarcoplasmic reticulum, mitochondrial electron transport, oxidative phosphorylation and calcium sequestration.

As mentioned above, the involvement of phosphodiesterase inhibition in the positive inotropic action of sulmazole was reported by various authors. A role of phosphodiesterase inhibition was also suggested by the potentiating action of sulmazole with regard to responses induced by isoprenaline in rabbit papillary muscles [2] and in isolated guinea-pig hearts [25]. However, the muscarinic agonist carbachol only partially attenuated the positive inotropic effects of sulmazole in papillary muscles [26, 27]. These results therefore suggest a partial contribution of phosphodiesterase inhibition in the positive inotropic effects of sulmazole. This interpretation is supported by the experimental data obtained with the stereoisomers of sulmazole. Although (+) and (−)sulmazole were roughly equipotent with respect to cAMP- and cGMP-phosphodiesterase inhibition in crude heart preparations of guinea-pig and swine, (+)sulmazole, both *in vivo* and *in vitro*, was a more potent positive inotropic agent (with higher intrinsic activity and a smaller ED_{50}) than (−)sulmazole. The difference in inotropic activity between the stereoisomers of sulmazole is therefore not related to phosphodiesterase inhibition. However, (+) and (−)sulmazole were also equipotent inhibitors of cAMP- and cGMP-phosphodiesterase in smooth muscle preparations *in vitro* and both produced equipotent hypotensive effects in two different *in vivo* animal preparations. Thus it is conceivable to explain the vasodilatory activities of the stereoisomers with

cAMP- and/or cGMP-phosphodiesterase inhibitory activities of the isomers [28, 29].

Inhibition of cardiac (Na–K)ATPase by sulmazole has been reported [2, 30]. A slight inhibition of cardiac (Na–K)ATPase was observed in the presence of 10 μmol/l of sulmazole (less than 10%) whereas the IC_{50} was about 1 mmol/l [2]. Similar observations have been made by Watanabe (personal communication) on canine cardiac sarcolemmal (Na–K)ATPase activity: an inhibition of about 9% at 0.1 mmol/l and about 26% at 0.3 mmol/l of sulmazole, respectively. In addition, a very low affinity of sulmazole was found for ^{3}H-ouabain specific binding sites in partially purified bovine adrenal medulla plasma membrane [30]. Our experiments extend this data; affinity for ^{3}H-ouabain specific binding sites was very low for (+)sulmazole in cat heart cell preparations whereas (−)sulmazole exhibited no measurable affinity up to 1 mmol/l. These observations were in agreement with the inhibitory activities on (Na–K)ATPase. (−)Sulmazole did not inhibit (Na–K)ATPase up to 1 mmol/l whereas (+)sulmazole showed some effect. The (+)isomer significantly inhibited (Na–K)ATPase at 0.1 mmol/l (less than 20%). However, these data do not explain the positive inotropic effects already observed at 10 μmol/l of (+)sulmazole. Nevertheless, a role for (Na–K)ATPase inhibition in the positive inotropic effects of (+)sulmazole cannot totally be excluded but might be relevant at concentrations higher than 0.3 mmol/l only.

A stimulation of myofibrillar calcium affinity in the presence of sulmazole was first described by Herzig *et al.* [7]. These investigators showed an additional increase in tension development in the presence of sulmazole at constant pCa in chemically skinned pig heart preparations. The pCa–tension curve was shifted to the left and this effect of sulmazole was dose-dependent. The observations were extended by Solaro and Rüegg [8] who reported an increase in calcium binding by cardiac myofibrillar protein and an activation of myofibrillar ATPase activity in the presence of sulmazole. The experiments additionally suggested an effect of sulmazole on the troponin–tropomyosin complex indicating an interaction with calcium binding sites. Other experiments using multiple superficial cells microinjected with the Ca^{2+}-sensitive bioluminescent protein aequorin also suggested that sulmazole increases the calcium sensitivity of myofibrils [9, 31]. However, as pointed out by these authors, a cAMP-dependent component antagonized by carbachol, was also demonstrated in the positive inotropic activity of sulmazole. Recent observations published by Ahn and coworkers [32] demonstrate that sulmazole was more potent as an inotropic agent than predicted on basis of its cAMP-phosphodiesterase inhibitory activity. A greater concentration of sulmazole was required for inhibition of cAMP-phosphodiesterase than for an inotropic effect suggesting that cAMP-phosphodiesterase inhibition alone may not account for its inotropic activity. In addition, these authors showed that for sulmazole the IC_{50} value was similar for heart crude phosphodiesterase and guinea-pig ventricular phosphodiesterase(III). Part of the positive inotropic activity of (±)sulmazole may be mediated by a cAMP-dependent mechanism. Both stereoisomers can contribute to this component. However, a substantial part of the inotropic activity of (±)sulmazole is cAMP-independent and it is tempting to explain this phenomenon by the contribution of the (+)stereoisomer of sulmazole to produce a pronounced increase in calcium sensitivity of myocardial fibres. Our experiments showed that the calcium sensitizing effect of sulmazole is a stereoselective event because only (+)sulmazole produced an unequivocal increase in additional tension development. The effects of (+)sulmazole were dose-dependent and more prominent at lower calcium concentrations similarly as described for the racemic mixture [7, 8]. Based upon the observations made by other investigators and our experiments with the stereoisomers of sulmazole, we propose that the difference in inotropic activity between the stereoisomers of sulmazole is brought about by the calcium sensitizing activity of the (+)isomer. However, at high concentrations (>0.3 mmol/l) other mechanisms of action of the (+)isomer cannot be excluded (e.g. (Na–K)ATPase inhibition).

Consequently, these experiments indicate that increasing calcium sensitivity of myofibrils substantially contributes to the positive inotropic activity of sulmazole. Increasing calcium sensitivity of myocardial contractile proteins is a mechanism of action which can evoke positive inotropic effects *in vitro* and *in vivo*. Such a mechanism although still not exactly defined, is a new approach for increasing cardiac contractility [6].

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