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Carane derivative stereoisomers of different local anaesthetic and antiplatelet activity similarly potentiate forskolin-stimulated cyclic AMP response and bind to β-adrenoceptors in the rat brain cortex

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Abstract

A carane derivative, KP-23 [*RS*](−)-4-(2-hydroxy-3)*N*-isopropylamino)-propoxyimino)-*cis*-carane, was earlier described as a potential local anaesthetic and antiplatelet agent, and the following studies revealed that its *R* and *S* stereoisomers, KP-23R and KP-23S, have different potencies in the infiltration anaesthesia and platelet aggregation tests. The effects of these stereoisomers on the cyclic AMP (cAMP) generating system and the displacement of [^{3}H]CGP 12177 (a β-adrenoceptor ligand) from its binding sites in the rat cerebral cortical tissue were investigated. The stereoisomers did not affect the basal cAMP level, but, at concentrations between 10^{-4} and 10^{-3} M, they elevated the forskolin-induced accumulation of cAMP with similar potency. The compounds displaced [^{3}H]CGP 12177, however the stereoisomer *R* was less potent than the racemic KP-23 and the *S* form ($K_i = 64.1 \pm 5.9$ nM, 161.1 ± 10 nM and 62.1 ± 5.6 nM for KP-23, KP-23R and KP-23S, respectively). The fact that the stereoisomers differed in both tests only slightly, if at all, suggests that their pharmacological effects are not related to the action on the β-adrenoceptor/adenylate cyclase system.

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Introduction

Local anaesthetics have anti-arrhythmic properties, and the compounds of this class with additional actions on the circulatory system may be useful clinical agents. For example, propranolol, combining beta-blocking and local anaesthetic activities, is one of the most important drugs for the prevention of cardiac infarct. Also, local anaesthetics acting additionally as platelet aggregation inhibitors may be potentially useful. A carane derivative, [*RS*](−)-4-(2-hydroxy-3)*N*-isopropylamino)-propoxyimino)-*cis*-carane (KP-23) is a compound of this type (Siemieniuk et al 1992). In our previous study, we reported that this compound facilitated the forskolin-induced accumulation of cyclic AMP (cAMP) in the cerebral cortical slices of the rat (Librowski et al 1994), and this property might be relevant to its action on platelet aggregation.

The racemic compound KP-23 consists of two stereoisomers of possibly diverse biological actions. They have identical absolute conformation of the lipophilic carane moiety, responsible for the interaction with the membrane, but opposite conformation at the chiral centre of the side chain, responsible for receptor activity (Pasenkiewicz-Gierula et al 2003). Since in the course of further studies the *R* and *S* stereoisomers of KP-23, KP-23R and KP-23S were synthesized (Lochynski et al 2002), it is now possible to compare the biological properties of such structures, which is interesting from both theoretical and practical points of view.

The stereoisomers indeed showed differences in biological activity. In the tests for local anaesthesia, the activity of the *R* stereoisomer, KP-23R, was significantly greater than that of the *S* isomer, KP-23S, and the activity of the former was comparable with that of the racemic KP-23 (Librowski et al 2001). In the tests for platelet aggregation KP-23S was more potent than both the enantiomer KP-23R and the racemate KP-23 (Librowski et al 2000).

In the present study, we investigated the action of both enantiomers on another biological activity of KP-23, the potentiation of forskolin-induced generation of cAMP,

and, because of the structural similarity of KP-23 compounds to propranolol, their interaction with β-adrenoceptors from the rat brain cortical membranes.

Materials and Methods

Chemicals

[^{3}H]CGP 12177 {(-(4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one)-hydrochloride} (specific activity 41 Ci mM^{-1}) was from Amersham (Buckinghamshire, UK); [8-^{14}C]cAMP (adenosine-3′,5′-monophosphate, ammonium salt) (specific activity 51 mCi mM^{-1}) and [2,8-^{3}H]adenine (specific activity 28 Ci mM^{-1}) were from DuPont NEN (Boston, MA); unlabelled cAMP (adenosine-3′,5′-monophosphate, sodium salt), forskolin and propranolol were from Sigma-Aldrich Co. (St. Louis, MO); Akwascynt was from BioCare (Warsaw, Poland); lidocaine (lignocainum hydrochloricum) was from Polfa (Warsaw, Poland). KP-23 and its stereoisomers, KP-23R and KP-23S, were synthesized at the Institute of Organic Chemistry and Biotechnology, Wroclaw University of Technology, Poland.

Animals

The animal experiments, in accordance to Polish law, were approved by the local Committee for Laboratory Animal Welfare and Ethics, and met the requirements of the European Council Guide for the Care and Use of Laboratory Animals (86/609/EEC). The experiments were carried out on cortical cerebral slices (cAMP assay) or cortical membrane preparation (receptor binding study) obtained from naive male Wistar rats, weighing 200–250 g, and kept under standard animal room conditions. The rats were killed by decapitation, their brains were excised and the cortices were dissected out.

cAMP assay

For preparation of slices, the tissue was sliced with a McIlwain tissue chopper (0.35 mm prisms), and the slices were suspended in O_2:CO_2 (95:5)-gassed glucose-containing modified Krebs-Henseleit medium (NaCl, 118 mM; KCl, 5 mM; $CaCl_2$, 1.3 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 25 mM; glucose, 11.7 mM; pH 7.4) at 37°C. This medium was used for all incubations. cAMP was assayed by the method of Shimizu et al (1969) with modifications as described elsewhere (Nalepa & Vetulani 1993). Briefly, after a 15-min adaptation period, the buffer was changed and [^{3}H]adenine was added to the incubation mixture in a final concentration of approximately 100 nM. After 45 min of incubation, the slices were washed, gravity-packed and distributed in 50-μL portions into vials containing 440 μL of the buffer. After 5 min of pre-incubation, the tested compound was added alone or together with the adenylate cyclase activator, forskolin. The compounds were added in a volume of 10 μL to obtain final concentrations of 1, 10, 50, 100, 200, 500, 1000 μM (tested drugs) or 1 μM (forskolin). The basal level was determined in the samples to which neither forskolin nor KP compounds were added. The incubation proceeded for 10 min, after which it was stopped with 550 μL of 10% trichloroacetic acid. The mixture was homogenized and centrifuged at 12 000 *g* for 10 min and the supernatant was decanted into test tubes. [^{3}H]cAMP was purified by co-chromatography with a tracer [^{14}C]cAMP using a two-column system (Dowex 50W × 4 and alumina) according to the method of Salomon et al (1974). The system was eluted with water and then the alumina column was eluted with 0.1 M imidazole solution. The final eluates were tested for radioactivity in a Beckman LS 6500 liquid scintillation counter ([^{14}C]/[^{3}H] channel; Beckman Coulter Inc., Fullerton, CA). All experiments were carried out in duplicate.

Receptor binding study

The membrane preparation (P2 fraction) was obtained by homogenization of the tissue in 20 volumes of 50 mM Tris-HCl buffer, pH 7.5, using a Polytron homogenizer set at 4, for 10 s, as described previously (Nalepa & Vetulani 1991). The supernatant after centrifugation of the homogenate (1000 *g*, 10 min) was homogenized again and centrifuged at 25 000 *g* for 30 min. The resulting pellet was resuspended and recentrifuged under the same conditions, and the preparation was stored at −20 °C until incubation. Homogenization and centrifugation were carried out at 0–4°C. Immediately before the assay, the pellet was reconstituted in 50 mM Tris-HCl buffer, pH 7.6, to obtain a final concentration of protein of approximately 1.0 mg mL^{-1}. The protein concentration was assayed using the method of Lowry et al (1951). β-Adrenergic receptor sites were labelled with the β-adrenergic receptor antagonist, [^{3}H]CGP 12177 (six concentrations ranging from 0.06 to 1.93 nM), and propranolol (10 μM) was used to define non-specific binding in the assay. The final incubation mixture contained 450 μL of membrane suspension, 50 μL of radioligand, and 50 μL of Tris-HCl buffer or of solution of displacer. The incubations were carried out at 25°C for 30 min in a water bath shaker, and were terminated by vacuum-assisted filtration through Whatman GF/C filters. The filters were then rinsed twice with 5-mL portions of ice-cold Tris-HCl buffer, placed in polyethylene minivials in 3 mL of Akwascynt solution, and counted for radioactivity in a Beckman LS 6500 liquid scintillation counter ([^{3}H] channel; Beckman Coulter Inc.).

In the study of direct interaction of KP-23 stereoisomers with β-adrenergic adrenoceptors, the membranes prepared from the rat cerebral cortex were used and inhibition of binding of the radioactive ligand by three tested compounds or propranolol was assessed. To characterize the interaction of tested compounds with β-adrenergic receptors, 13 concentrations of the compounds ($10^{-11} - 10^{-4}$ M) in the presence of 0.43 nM of [^{3}H]CGP 12177, and in a parallel experiment, the same 13 concentrations of a specific β-adrenoceptor ligand, propranolol, were tested. All experiments were carried out in triplicate.

Calculations

The B_{max} (fmol (mg protein)$^{-1}$) and K_D (nM) values were calculated from the saturation binding isotherm with

the formula $Y = B_{max} * X/(K_D + X)$. The K_D values were used for the calculation of K_i values in the subsequent experiments. K_i values were calculated from a sigmoid curve with the formula $K_i = IC50/(1 + L/K_D)$, where L is the concentration of the radioligand and K_D is its dissociation constant (Cheng & Prusoff 1973). The GraphPad Prism program was used for all calculations.

The cAMP (% conversion) data are percentages of conversion of [^{3}H]adenine to [^{3}H]cAMP ± s.e.m. after subtraction of basal values and represent net stimulation (increase over the basal level, which was 0.07 ± 0.015, n = 5).

Statistical analysis

The results were evaluated with one-way or two-way analysis of variance followed by individual comparisons with the Fisher's least significant difference test using Statistica 6.0 software (Statsoft Inc., Tulsa, OK).

Results

Effects of KP-23 stereoisomers on basal and forskolin-stimulated cAMP responses

The KP-23 compounds at concentrations above 100 μM dose-dependently enhanced the forskolin-induced accumulation of cAMP in the cerebral cortical slices (Figure 1). Although the trend of the dose–response curves suggests that racemic KP-23 was more effective than its stereoisomers, as a significant effect was induced by a concentration of 200 μM, the analysis of variance demonstrated no significant differences between the drugs. The two-way analysis of variance for KP-23 compounds (*RS*, *R*, *S*) over the dose range from 100 to 1000 μM showed no significant difference between KP-23 isomers ($F = 2.067$, df 2/44), with significant effect of dose ($F = 5.51$, df 3/44, $P = 0.002$), with no interaction ($F = 0.31$, df 6/44). Lidocaine, which served as a reference compound in tests for local anaesthetic properties of KP-23 in the previous study (Librowski et al 2001), was also tested in the present study and was found not to affect the forskolin-induced accumulation of cAMP. Neither compound affected the basal accumulation of cAMP (results not shown).

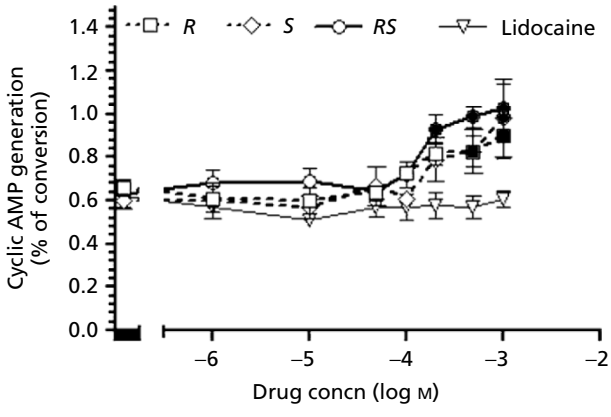

Figure 1 The effect of KP-23 stereoisomers on forskolin-stimulated cyclic AMP generation in rat cortical slices in-vitro. Values indicated by closed symbols are significantly different ($P < 0.01$) compared with the control values (in the absence of KP-23 compounds).

Effects of KP-23 stereoisomers on β-adrenoceptor binding sites in rat cerebral cortex

To characterize β-adrenergic receptor binding sites, six concentrations of [^{3}H]CGP 12177, ranging from 0.11 nM to 3.44 nM were used, and propranolol or KP-23 compounds at a final concentration of 10 μM served as displacers. The one-way analysis of variance demonstrated significant differences between the groups: $F_{3/20} = 20.4$ for B_{max}, and $F_{3/20} = 4.3$ for K_D.

Saturation binding analysis with [^{3}H]CGP 12177 in the absence or presence of one concentration (10 μM) of propranolol and KP-23 compounds revealed that B_{max} values for propranolol were significantly higher, while the differences between racemic and optically active KP-23 compounds were much smaller, although the B_{max} for KP-23S was significantly lower (by approx. 25%) than for the racemate and *R* isomer (Table 1).

The results suggest that the tested compounds may detect either a subpopulation or a different population of β-adrenoceptor than propranolol does. This is supported by the fact that K_D values for KP-23 compounds (which were similar) were significantly lower than the K_D value for propranolol (Table 1).

To characterize the interaction of KP-23 stereoisomers with β-adrenergic receptors, we tested 13 concentrations of the compounds in the presence of [^{3}H]CGP 12177, and the same 13 concentrations of a specific ligand, propranolol. All compounds displaced specifically the β-adrenoceptor ligand, [^{3}H]CGP 12177, from its binding sites (Figure 2), but they were much less potent (15–38 times) than propranolol in this respect. The racemic KP-23 and the KP-23S stereoisomer were more potent (by a factor of 2.5) than the KP-23R stereoisomer. The respective K_i values were 4.2 ± 0.3 nM for propranolol, 64.1 ± 5.9 nM for KP-23, 62.1 ± 5.6 nM for KP-23S, and 161.1 ± 10.4 nM for KP-23R.

Discussion

Local anaesthetic (Librowski et al 2001) and antiplatelet (Czarnecki et al 1992) activities are prominent and potentially

Table 1 Effects of KP-23 stereoisomers on β-adrenoceptor binding sites in rat cerebral cortex

Compound	B_{max} (fmol (mg protein)$^{-1}$)	K_D (nM)
Propranolol	103.2 ± 5.8*	0.62 ± 0.07*
KP-23RS	61.5 ± 6.3	0.39 ± 0.06
KP-23S	48.0 ± 2.8†	0.40 ± 0.05
KP-23R	69.5 ± 5.2	0.34 ± 0.04

*$P < 0.01$, significantly different compared with KP-23 compounds; $^{\dagger}P < 0.01$, significantly different compared with KP-23R; n = 6.

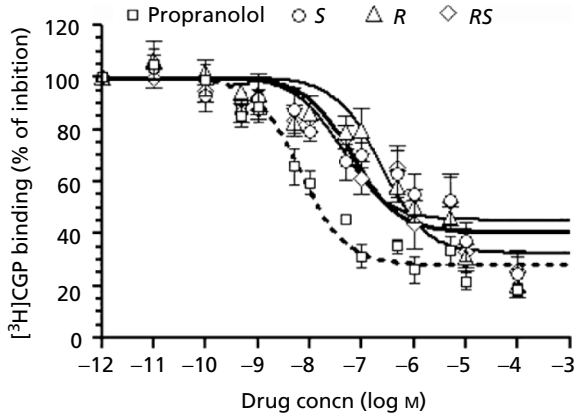

Figure 2 Displacement by KP-23 compounds and propranolol of [^{3}H]CGP 12177 from its binding sites (n = 6).

useful properties of the carane derivatives investigated in the present study. The local anaesthetic action of similar natural carane derivatives has found application in clinical practice (Parturier-Albot & Veyne 1960; Gaudin 1963; Baronet & Gaudin 1966; Wanet 1979), suggesting a similar potential for KP compounds.

The compounds investigated in the present study may be regarded as derivatives of propranolol (Siemieniuk et al 1992), one of the most commonly used antihypertensive drugs. In addition to its β-blocking properties, propranolol is also a potent local anaesthetic (Paradise & Stoelting 1966) and, similar to another β-blocker, sotalol, may liberate hypotensive autacoids from the endothelium (Korbut et al 1998). Similar to KP compounds, propranolol seems to display antithrombotic activity, as it inhibits ADP-, collagen- or serotonin-stimulated platelet aggregation (Weksler et al 1977; Gasser & Betterridge 1991; Gruszecki et al 2001).

Similar to propranolol (Howe & Shanks 1966), KP compounds exist in stereoisomeric forms. Although their structure contains four chiral centres, the stereospecific synthesis results in only two stereoisomers, with differences limited to the conformation at the side chain.

Stereospecificity may result in changes in biological activity and thus offers an insight into the conformation–activity relationship. Propranolol stereoisomers may differ in their pharmacological action in some respects (Barret & Cullum 1968). Thus, the stereoisomers act similarly on membranes, being equipotent anti-arrhythmic agents as tested in the ventricular fibrillation in rats (Töelg et al 1997), whereas only the *S* isomer is active in binding to the receptor (Mancinelli et al 1991; Stone et al 1995). There were also some differences in the pharmacokinetics of *R* and *S* propranolol (Bleske et al 1995). The differences in the properties of individual KP-23 stereoisomers seemed, therefore, to warrant investigation.

In earlier pharmacological experiments, the KP stereoisomers were found to act differently (Librowski et al 2000, 2001). The antiplatelet activity of KP-23S was more potent than that of the racemate KP-23 and the isomer KP-23R (Librowski et al 2000), while in the tests for local anaesthesia, the *R* stereoisomer and racemate were almost equipotent and both were much more effective than the *S* stereoisomer (Librowski et al 2001). The present study intended to find out whether these differences are reflected in the action of KP compounds at the receptor and intracellular levels. We aimed to compare the aspects of action of KP stereoisomers possibly associated with their antiplatelet activity. This action of KP compounds may be related to the stimulation of generation of cAMP (Feijge et al 2004), and, therefore, the effect of KP-23 stereoisomers on the cAMP generating system was investigated. The generation of cAMP depends on the activity of adenylate cyclase, which is regulated by β-adrenoceptor and by extra-receptor mechanisms. In the present study, we investigated whether the KP enantiomers bind to adrenoceptors and how they influence the response of adenylate cyclase to direct stimulation by forskolin.

As in our previous study (Librowski et al 1994), we found that KP-23 potently enhanced the effect of forskolin on generation of cAMP, although the compound by itself did not affect the activity of adenylate cyclase, leaving unchanged the basal accumulation of cAMP. Both *R* and *S* enantiomers acted similarly to the racemate, and although the shapes of the concentration–effect curves differed slightly, the analysis of variance revealed no significant differences between the compounds. The discrepancy between the difference in biological actions of KP enantiomers and the similarity of their action on the response to forskolin suggests that the action of KP compounds on forskolin-induced stimulation of adenylate cyclase activity is irrelevant to their investigated pharmacological effects. Evidently, the enhancement of the effect of forskolin is not necessary for anaesthetic action, as lidocaine was ineffective in this respect.

Forskolin, a diterpene compound, is a direct activator of adenylate cyclase, the enzyme generating cAMP (Hurley 1998). The action of the generated second messenger is limited by phosphodiesterase, an enzyme whose activity depends on calcium and calmodulin (Kakkar et al 1999). Several local anaesthetics, including propranolol, are calmodulin antagonists (Volpi et al 1981), and, therefore, they may enhance the effect of forskolin on the generation of cAMP. It is highly probable that the present reported action of KP-23 compounds, which share with propranolol several pharmacodynamic properties, may also be related to their inhibitory effect on phosphodiesterase. The observed action of KP-23 compounds is non-stereospecific, and it is worth noting that the effect of propranolol on calmodulin-stimulated phosphodiesterase was also non-stereospecific (Volpi et al 1981).

Since cAMP inhibits platelet aggregation (Winther & Trap-Jenssen 1988), it is tempting to speculate that the potentiating effect of KP-23 compounds on forskolin-induced cAMP may be responsible, at least in part, for their antiaggregatory action (Librowski et al 2000). cAMP regulates the uptake of free intracellular calcium (to the platelet calcium storage pool) and inhibits phospholipase A2, an essential enzyme in the arachidonic pathway in blood platelets (Puri 1998), and β-adrenoceptor-blocking drugs that share chemical structure with propranolol were reported to inhibit the platelet cytosolic phospholipase A2 and the phospholipase A2-related liberation of arachidonic acid from membrane phospholipids (Nosal & Jancinova 2002).

The primary chemical structure of propranolol and related compounds facilitates their functioning as cationic amphiphilic drugs (Liscovitch & Lavie 1991). KP-23 compounds are structurally related to propranolol and also have the characteristics of cationic amphiphilic drugs (Pasenkiewicz-Gierula et al 2003). Similar to other cationic amphiphilic drugs, KP-23 compounds intercalate easily into the phospholipid bilayer (Pasenkiewicz-Gierula et al 2003). The disturbance of the membrane structure by cationic amphiphilic drugs results in changes in the membrane properties, such as its fluidity, order and charge (Toplak et al 1990; Wiktorek-Wojcik et al 1997; Strugala et al 2000) and affects the intracellular signalling, in a non-specific manner. Cationic amphiphilic drugs were reported to influence the intracellular calcium and the activity of enzymes involved in calcium homeostasis (Bobeszko et al 2000, 2002), which is the determining factor for blood platelet activation (Rao 1993). This mechanism is non-stereospecific and, hence, the stereoisomers did not differ in their action in this respect.

The important question is which mechanism may explain the antiplatelet action of KP-23 compounds. The fact that the difference in binding of both KP-23 stereoisomers to β-adrenoceptors is rather moderate and they similarly enhance the forskolin-induced cAMP accumulation, while their anti-aggregatory effects and local anaesthetic activities clearly differ, suggests that the interesting pharmacological properties of the KP-23 isomers are not related to their action on the β-adrenoceptor system.

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