Inhibitory Effects of Pentacaine and Some Related Local Anaesthetics on Rat Hepatic Adenylate Cyclase

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Abstract. In the present study effects of a new local anaesthetics, pentacaine (trans-2-pyrolidinocyclohexylester of 3-pentyloxyphenylcarbamic acid), and of some chemically related compounds on rat hepatic adenylate cyclase activity were studied under various experimental conditions. As compared with tetracaine, the local anaesthetics tested showed stronger inhibitory effects, regardless of the type of stimulating agents used to activate adenylate cyclase. The most potent effect was observed with pentacaine. Its inhibitory effects on glucagon, guanylylimidodiphosphate (Gpp/NH/p), sodium fluoride or forskolin stimulated activity suggest that it may directly act on the catalytic unit of adenylate cyclase. The same conclusion can be drawn based on its inhibitory effects on adenylate cyclase, regardless ATP concentrations used as the enzyme substrate, and on octylpyranoside solubilized enzyme activated by preincubation of the enzyme preparation with Gpp/NH/p. Structure-activity studies have suggested that the pentacaine molecule as a whole and none of its parts alone or its analogs are responsible for the inhibitory effect. However, the inhibitory effects of these compounds on the rat adenylate cyclase activity do not correlate with their local anaesthetic properties. The possibility of using adenylate cyclase inhibitors to decrease cyclic AMP production under pathological conditions, like in cholera, known to be due to a high adenylate cyclase activity, is discussed.

Key words: Adenylate cyclase — Pentacaine — Tetracaine — Local anaesthetics — Glucagon — Forskolin — Octylpyranoside — Cyclic AMP

Introduction

One of the important contributions to the study of the molecular architecture and function of the adenylate cyclase system is the adoption of the concept of the dynamic and fluid nature of cell membranes (Cuatrecasas 1975). The application of

this mobile receptor hypothesis to the adenylate cyclase system has prompted the testing of many compounds, which are known to change plasma membrane fluidity, on this system. These compounds include local anaesthetics (Voeikov and Lefkowitz 1980; Seeman 1972), aliphatic alcohols and other organic solvents (Hynie and Klenerová 1980), as well as a number of other compounds (Ross and Gilman 1980).

Tetracaine and other local anaesthetics have multiple effects on the adenylate cyclase system from various sources (Voeikov and Lefkowitz 1980; Housley et al. 1980; Voorheis and Martin 1982; Hepp et al. 1978). In the present study effects of a new local anaesthetic, pentacaine, and some chemically related compounds on rat hepatic adenylate cyclase are described. Moreover, an attempt was made to analyze the mode of the inhibitory action of pentacaine which substantially differs, as for its quantitative parameters, from effects of other local anaesthetics tested.

Materials and Methods

Animals. Rats used in this study (Wistar strain), weighing 200—300 g and fed ad libitum, were obtained from Velaz, Prague.

Enzyme preparations

Adenylate cyclase preparation. The enzyme preparation (Hynie and Klenerová 1980) for adenylate cyclase activity determination was obtained by gentle homogenization of 0.5 g rat liver in 5 ml ice-cold 75 mmol/l Tris-HCl buffer, pH 7.5, with 12.5 mmol/l $MgCl_2$ and 0.5 mmol/l EDTA in Dounce all glass tissue grinder (Kontes Glass, Vineland, N. J.). After ten strokes with pestle A and ten strokes with pestle B, the homogenate was filtered through two layers of cheescloth and centrifuged for two min at $900 \times g$. The sediment was twice washed and finally suspended in 5 ml of the same homogenization medium. This preparation was considered as a crude plasma membrane fraction.

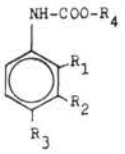
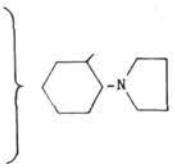
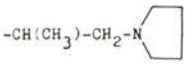
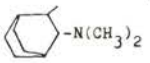
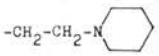
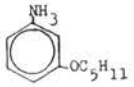
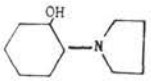
Preincubation of adenylate cyclase preparations. In experiments with preincubation (Hynie and Klenerová 1980) 1 ml of crude plasma membrane fraction was incubated with the respective additions at 37 °C for 5 min. The samples were then cooled, centrifuged at 0—4 °C for 5 min at $900 \times g$, resuspended in 1 ml of the homogenization medium described above, and the whole washing procedure was repeated 2 times.

Solubilization of adenylate cyclase preparations. Crude plasma membrane fraction from rat liver was activated by preincubating the enzyme preparation with 0.1 mmol/l of Gpp/NH/p as described above. Then, the enzyme was solubilized by 30 mmol/l of octyl-β-*D*-glucopyranoside (Scotto and Swislocki 1982), centrifuged for 1 hour at $100{,}000 \times g$ and filtered through a Synpor filter (0.45 μm). Adenylate cyclase activity was assayed in the supernatant; the final concentration of octyl-β-*D*-glucopyranoside in the assay mixture was 6 mmol/l.

Adenylate cyclase assay

Adenylate cyclase activity was determined by a method with adenosine 5'-(α-^{32}P) triphosphate used as substrate, and the product was isolated on aluminium oxide (Hynie and Klenerová 1980). In a total volume of 50 μl, the assay contained (unless indicated otherwise): 0.1 mmol/l ATP, approximately

Table 1. Chemical structure of some new local anaesthetics and their analogs

Name (symbol)				
	R_1	R_2	R_3	R_4
Pentacaine (P_1)	—H	$-OC_5H_{11}$	—H	
— (P_1-para)	—H	—H	$-OC_5H_{11}$	
— (P_2)	—H	$-OC_3H_7$	—H	
— (K_{3002})	—H	—H	—H	
— (P_3)	—H	$-OC_5H_{11}$	—H	
— (TB-7)	$-OC_6H_{13}$	—H	—H	
Carbizocaine (—)	$-OC_6H_{13}$	—H	—H	$-CH(CH_3)-CH_2-N(C_2H_5)_2$
Heptacaine (—)	$-OC_7H_{15}$	—H	—H	
(POA)				
(TPC)				

$5—10 \times 10^5$ cpm of ^{32}P-α-ATP), 30 mmol/l Tris-HCl buffer, pH 7.5, 0.2 mmol/l cyclic AMP, 5 mmol/l $MgCl_2$, 1 mmol/l KCl, 0.2 mmol/l EDTA, 5 mmol/l phosphoenolpyruvate, myokinase 20 μg/ml and phosphoenolpyruvate kinase 40 μg/ml, 20 μl enzyme preparation (40—80 μg protein) and the respective additions. After 10 min of incubation the product was separated as described earlier (Hynie and Klenerová 1980). ^{32}P radioactivity was measured by Cerenkov's radiation in aqueous solution of umbelliferone (30 mg/l).

Protein determination

Protein content in enzyme preparations was determined by the method of Lowry et al. (1951).

Mathematical analysis

Results of adenylate cyclase activity measurements were expressed either in absolute values of ^{32}P-cyclic AMP formed or as percentages of the respective adenylate cyclase activity. Experiments were

Table 2. Inhibitory effects of various local anaesthetics on rat hepatic adenylate cyclase

Additions to adenylate cyclase assay	Adenylate cyclase activity[1]		
	Glucagon (10^{-6} mol/l)	Gpp/NH/p (10^{-4} mol/l)	NaF (10^{-2} mol/l)
Local anaesthetics (2×10^{-3} mol/l):	Percentage of control values ± SEM ($n = 3$)		
Tetracaine	102 ± 2	125 ± 16	122 ± 2
Heptacaine	46 ± 4	73 ± 8	89 ± 6
TB-7	44 ± 3	75 ± 6	81 ± 3
Carbizocaine	43 ± 6	73 ± 3	70 ± 6
Pentacaine	1 ± 1	5 ± 1	11 ± 3

[1]) Adenylate cyclase activity in crude plasma membranes of rat livers expressed in pmol of cAMP per mg protein. 10 min^{-1} ± SEM: basal = 35 ± 3; glucagon = 324 ± 12; Gpp/NH/p = 160 ± 16; NaF = 241 ± 10.

performed in duplicate or triplicate with three different batches of adenylate cyclase preparation. A representative experiment is presented. Mean values ±S.E.M. are given.

Drugs used

^{32}P-α-ATP was prepared in this laboratory by the method of Symons (1977) with the use of Cl^--free $H_3{}^{32}PO_4$ (Academy of Sciences, GDR). Guanylylimidodiphosphate (Gpp/NH/p) was from Boehringer Mannheim GmbH, forskolin was a product of Calbiochem and octyl-β-*D*-glucopyranoside was prepared by M. Černý, Faculty of Natural Sciences, Prague. All other chemicals were from sources described in previous work (Hynie and Klenerová 1980).

Local anaesthetics. All the local anaesthetics used, with the exception of tetracaine and cinchocaine (Spofa, $ČSL_3$, Czechoslovakia), were prepared and kindly donated by L. Beneš and coworkers (Institute of Experimental Pharmacology, Centrum of Physiological Science, Slovak Academy of Sciences, Bratislava). Table 1 shows the structure, names or symbols of the anaesthetics used. Also two fractions of pentacaine (trans-2-pyrolidinocyclohexylester of 3-pentyloxyphenylcarbamic acid) m-pentyloxaaniline (POA) and trans-2-/1-pyrolidinyl/cyclohexanol (TPC) (Beneš et al. 1969) were used.

Results

Inhibitory effects of various local anaesthetics on rat hepatic adenylate cyclase

Comparison of effects of tetracaine with those of some new local anaesthetics

In a first series of experiments, effects of tetracaine on rat hepatic adenylate cyclase activity stimulated by glucagon, Gpp/NH/p or sodium fluoride (Table 2) were compared with those of four new local anaesthetics. Tetracaine in a concentration of 2 mmol/l had no effect on the enzyme preparation stimulated by glucagon, or it

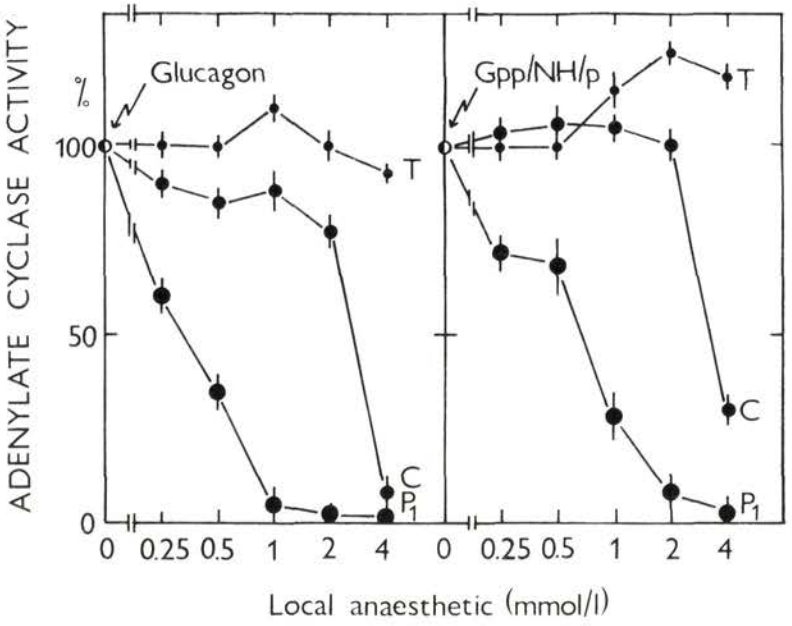


Fig. 1. Comparison of the effects of tetracaine (T), cinchocaine (C), and pentacaine (P_1) on rat hepatic adenylate cyclase activity. Crude plasma membrane fraction was prepared as described in Methods. Adenylate cyclase activity in pmol . mg protein^{-1}. 10 min^{-1} ± SEM: Basal = 23 ± 2; glucagon, 1 μmol-/l = 229 ± 15; Gpp/NH/p, 10 μmol/l = 121 ± 12; (n = 2).

slightly increased adenylate cyclase activity stimulated by Gpp/NH/p or sodium fluoride. All other local anaesthetics tested showed inhibitory action on adenylate cyclase activity in crude plasma membranes of rat livers. The activity stimulated by glucagon was inhibited more intensely than that stimulated by Gpp/NH/p or sodium fluoride. The inhibitory effects of pentacaine were much stronger than those of other local anaesthetics tested, although their local anaesthetic properties are comparable with those of pentacaine (Švec et al. 1976; 1979; Beneš et al. 1969; Beneš, personal communication). This finding prompted us to analyze further the effects of this new local anaesthetic.

A comparison of inhibitory effects of increasing concentrations of pentacaine with those of standard local anaesthetics, tetracaine and cinchocaine, on rat hepatic adenylate cyclase activity is shown in Fig. 1. The strongest inhibition of both glucagon and Gpp/NH/p-activated enzyme was observed with pentacaine. Cinchocaine showed inhibitory effects at concentrations as high as 4 mmol/l, while tetracaine was ineffective even at this concentration.

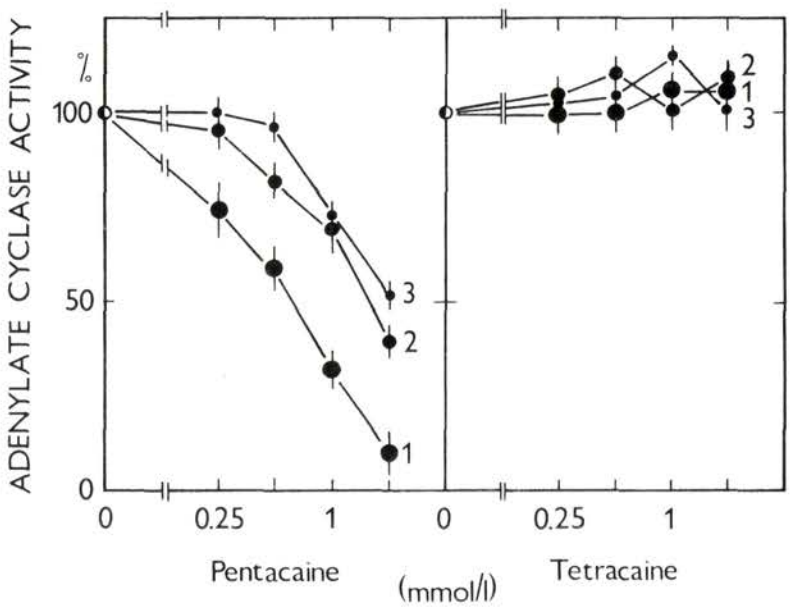


Fig. 2. Effects of pentacaine and tetracaine on rat hepatic adenylate cyclase activity stimulated by glucagon (1), Gpp/NH/p (2), or preincubated for 5 min with Gpp/NH/p (3). Adenylate cyclase preparation as in Fig. 1. Enzyme preincubation with Gpp/NH/p, 0.1 mmol/l as described in methods. Adenylate cyclase activity in pmol . mg protein^{-1}. 10 min^{-1} ± SEM: Basal = 28 ± 4; glucagon, 1 μmol/l = 160 ± 8; Gpp/NH/p, 0.1 mmol/l = 204 ± 16; enzyme preincubated with Gpp/NH/p, 0.1 mmol/l = 281 ± 17; (n = 2).

Intensity of inhibitory effects of local anaesthetics in relation to the type of adenylate cyclase activation

Data shown in Table 2 and Fig. 1 indicate that the intensity of inhibitory effects of local anaesthetics depends on the type of adenylate cyclase activation. Glucagon-stimulated adenylate cyclase is inhibited stronglier than the activity stimulated by Gpp/NH/p or sodium fluoride. A comparison of the inhibitory effects of pentacaine and tetracaine on rat adenylate cyclase activity stimulated by glucagon, Gpp/NH/p or by enzyme preincubation with Gpp/NH/p (nonhydrolysable GTP derivative) which induces enzyme into the persistent active state (Cuatrecasas et al. 1975) is shown in Fig. 2. Tetracaine in concentrations ranging from 0.25 to 2 mmol/l had no inhibitory effect. When expressed as percentual values, the inhibitory effects of similar concentrations of pentacaine were much weaker when Gpp/NH/p was used to stimulate the enzyme as compared to glucagon-stimulated enzyme. Gpp/NH/p-preincubated enzyme showed the least inhibition.

Table 3. Effects of pentacaine on rat hepatic adenylate cyclase during the enzyme preincubation

Additions to adenylate cyclase assay	Additions during enzyme preincubation			
	None	Pentacaine (2 mmol/l)	Gpp/NH/p (10 μmol/l)	Gpp/NH/p + Pentacaine
	cAMP, pmol . mg protein^{-1} . 10 min^{-1} ± SEM ($n = 3$)			
Basal	10 ± 2	35 ± 2	140 ± 10	45 ± 5
Glucagon, 1 μmol/l	170 ± 10	35 ± 4	200 ± 12	40 ± 3
Gpp/NH/p, 10 μmol/l	140 ± 9	70 ± 4	230 ± 8	60 ± 10

Effect of preincubation with pentacaine on adenylate cyclase activity

The addition of pentacaine (2 mmol/l) to the enzyme preparation preincubated for 5 min in the absence or presence of Gpp/NH/p produced two typical effects (Table 3). First, the enzyme preparation preincubated with pentacaine alone was not activated by subsequent addition of glucagon or Gpp/NH/p to the adenylate cyclase assay. Second, the addition of pentacaine to Gpp/NH/p during enzyme preincubation prevented the induction of the persistent active state of the enzyme. Also, the enzyme was not further activated by the addition of stimulators to the adenylate cyclase assay.

A comparison of the effects of pentacaine with those of tetracaine during enzyme preincubation on subsequent stimulation of adenylate cyclase activity by various agents, including sodium fluoride and forskolin, the latter being considered as acting directly on the catalytic unit of adenylate cyclase (Seamon et al. 1981) is shown in Table 4. The addition of tetracaine during enzyme preincubation reduced the subsequent adenylate cyclase activation by about 30 %, and it also prevented, to a similar degree, the persistent activation of the enzyme during its preincubation with Gpp/NH/p. Under similar experimental conditions, the effect of pentacaine was much stronger, producing about 70—90 % inhibition of both the Gpp/NH/p preincubation-induced persistent activation of adenylate cyclase and the subsequent enzyme activation by various drugs with stimulatory effect on different levels of the adenylate cyclase system.

Analysis of the mode of the inhibitory action of pentacaine on adenylate cyclase

Relationship between ATP concentration and the inhibition of adenylate cyclase by pentacaine

The inhibitory effect of pentacaine was tested on adenylate cyclase activity stimulated by sodium fluoride or forskolin in the presence of increasing concentra-

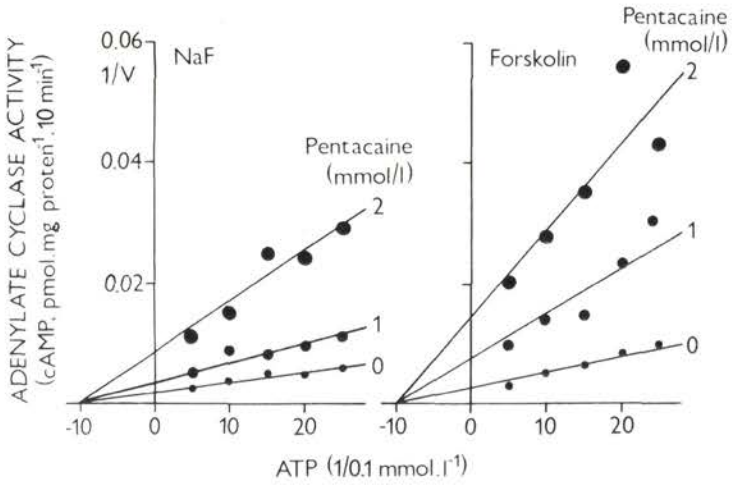


Fig. 3. Lineweaver-Burk plot of adenylate cyclase activity in rat liver in the presence of increasing concentrations of ATP as substrate and pentacaine as enzyme inhibitor. *Left:* enzyme stimulated by sodium fluoride, 10 mmol/l; *right:* enzyme stimulated by forskolin, 10 μmol/l.

tions of ATP (0.04—0.2 mmol/l). The data are presented in a double reciprocal Lineweaver-Burk plot (Fig. 3). The inhibitory effect of pentacaine showed a noncompetitive type of antagonism on adenylate cyclase stimulated by both sodium fluoride and forskolin.

The effect of pentacaine on solubilized adenylate cyclase

Adenylate cyclase in persistent active state, induced by the enzyme preincubation with Gpp/NH/p, was solubilized with octyl-β-*D*-glucopyranoside and the activity of this preparation was estimated in the presence of pentacaine or tetracaine (Fig. 4). Pentacaine inhibited the activity of the solubilized adenylate cyclase similarly as it was the case with the membrane-bound enzyme, while tetracaine had no similar effect.

Structural requirements for the inhibitory effects of pentacaine and its derivatives

Fig. 5 shows that pentacaine and its analog lacking the cyclohexenyl group (P_3) had very strong inhibitory effects on glucagon-stimulated adenylate cyclase activity in crude plasma membrane fraction. The unsubstituted analog K_{3002} had no inhibitory effect (data not shown), and the pentacaine analog with a shorter alkyloxysubstituet $-OC_3H_7$ (P_2) showed much weaker inhibitory effects, similar to those

Table 4. Effects of tetracaine and pentacaine on rat hepatic adenylate cyclase during the preincubation in the absence or presence of Gpp/NH/p

Additions to adenylate cyclase assay	Additions during enzyme preincubation					
	None	Tetracaine (2 mmol/l)	Pentacaine (2 mmol/l)	Gpp/NH/p (0.1 mmol/l)	Gpp/NH/p + tetracaine	Gpp/NH/p + pentacaine
	cAMP, pmol . mg protein^{-1} . 10^{-1} ± SEM ($n = 3$)					
None	6 ± 1	3 ± 1	4 ± 1	316 ± 10	236 ± 15	46 ± 5
Glucagon, 1 μmol/l	93 ± 5	62 ± 3	6 ± 2	281 ± 12	204 ± 2	42 ± 2
Gpp/NH/p, 0.1 mmol/l	102 ± 4	79 ± 10	13 ± 2	281 ± 10	239 ± 12	34 ± 4
Forskolin, 10 μmol/l	35 ± 3	29 ± 4	4 ± 1	421 ± 14	365 ± 30	29 ± 6
NaF, 10 mmol/l	111 ± 10	61 ± 8	24 ± 2	310 ± 8	239 ± 8	38 ± 5

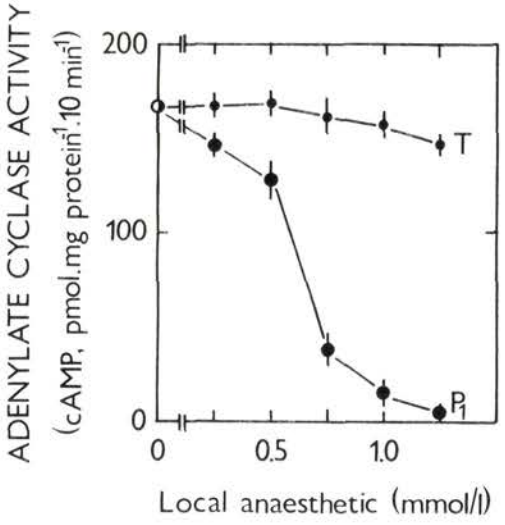


Fig. 4. Effects of local anaesthetics on solubilized hepatic adenylate cyclase activity. Enzyme was preincubated with Gpp/NH/p (0.1 mmol/l) for 5 min, solubilized by 30 mmol/l octyl-β-*D*-glucopyranoside, centrifuged at 100,000 × *g* and filtered through Synpor 0.45 μm; adenylate cyclase activity was assayed in the presence of 6 mmol/l of octyl-β-D-glucopyranoside. T = tetracaine, P_1 = pentacaine.

produced by cinchocaine. The same sequence of potencies of these drugs could also be shown on Gpp/NH/p-stimulated enzyme preparation (data not shown). Prolongation of alkylsubstituents by itself did not raise the inhibitory effects on adenylate cyclase as shown by effects of three drugs in Table 2. A comparison of the inhibitory effects of pentacaine with those of its para-OC_5H_{11} derivative shows that both compounds had practically identical inhibitory effects on both the

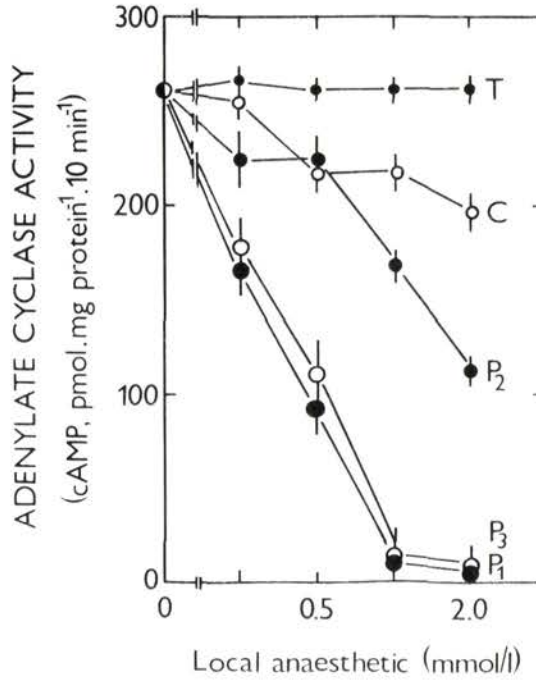


Fig. 5. Comparison of the inhibitory effects of various local anaesthetics and their derivatives on rat hepatic adenylate cyclase stimulated by glucagon (1 μmol/l). For abbreviations and structure of compounds, see Table 1, C = cinchocaine, T = tetracaine. Glucagon-stimulated cyclase activity in pmol.mg protein^{-1}. 10 min^{-1} = 260 ± 18 (n = 4).

glucagon-stimulated (Fig. 6) and the Gpp/NH/p-stimulated adenylate cyclase activity (data not shown).

Effects of pentacaine fragments

Effects of the pentacaine fragments POA and TPC (see Table 1) on glucagon-stimulated adenylate cyclase activity were also studied (Fig. 6). When added alone, these compounds had no inhibitory effects comparable to those of pentacaine on glucagon- (Fig. 6) or Gpp/NH/p-stimulated adenylate cyclase activity. A combined application of these fragments had no significant inhibitory effect either (data not shown).

Discussion

Interesting observations have recently been reported concerning the effects of

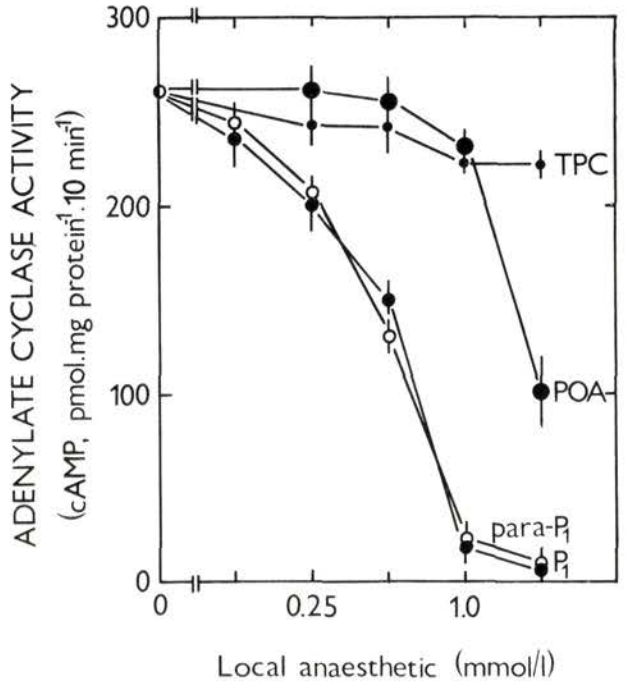


Fig. 6. Effects of pentacaine, its fragments POA and TPC (see Table 1), and para-pentacaine (para-P_1) on hepatic adenylate cyclase stimulated by glucagon, 1 μmol/l. Adenylate cyclase activity as in Fig. 5.

various local anaesthetics on the adenylate cyclase system in enzyme preparations from various sources (see Introduction). In the present study effects of some new local anaesthetics were examined on the rat liver adenylate cyclase system. The results have shown that, as compared with tetracaine, the local anaesthetics shown in Table 1 have much stronger inhibitory effects; pentacaine appeared to be the most potent drug, and its effects were therefore analyzed more thoroughly.

The adenylate cyclase system in crude plasma membranes prepared from rat livers responds to tetracaine and cinchocaine much weaker than the enzyme from erythrocyte membranes (Voeikov and Lefkowitz 1980). However, rat hepatic adenylate cyclase is sensitive enough to the inhibitory action of some new local anaesthetics which show properties different from those described for classical local anaesthetics. Inhibitory effects of the drugs tested, on rat liver adenylate cyclase activity do not closely correlate with their local anaesthetic potencies (Švec et al. 1976, 1979; Beneš et al. 1969; Beneš, personal communication). Moreover, as it will be discussed bellow, their mechanism of action is different from that described for tetracaine and cinchocaine in adenylate cyclase preparation from erythrocyte membranes.

In a first series of experiments effects of pentacaine and some other new local anaesthetics were tested on rat hepatic adenylate cyclase, stimulated by glucagon, Gpp/NH/p or sodium fluoride. Quite exceptional was the effect of pentacaine which produced nearly complete inhibition of adenylate cyclase activity regardless of the stimulator used. With this preparation, tetracaine had no inhibitory effect, and one of the most potent local anaesthetics tested, TB-7, also showed a much weaker inhibitory effect on adenylate cyclase than pentacaine. This prompted us to analyze more closely the inhibitory effects of pentacaine on hepatic adenylate cyclase.

In a second series of experiments inhibitory effects of pentacaine were studied on adenylate cyclase stimulated by various means. The most strongest inhibitory effect was observed with hormone-stimulated enzyme, while enzyme stimulated by sodium fluoride or Gpp/NH/p was less strongly inhibited. The weakest inhibition was observed with the enzyme in persistent active state (Cuatrecasas et al. 1975) following preincubation with Gpp/NH/p. These results suggest that pentacaine acts on several sites of adenylate cyclase complex. However, the general inhibitory effects regardless of the way of stimulation of adenylate cyclase also suggest a direct inhibitory effect of pentacaine on the catalytic unit of this enzyme. This suggestion is also supported by the observation that pentacaine inhibits the enzyme stimulated by forskolin (an agent considered to act directly on the catalytic unit (Seamon et al. 1981) (Table 4 and Fig. 3), and the solubilized enzyme (Fig. 4). The non-competitive type of the adenylate cyclase inhibition by pentacaine in relation to ATP concentration (Fig. 3) suggest that this local anaesthetic acts on the allosteric site of the catalytic unit.

In the last series of experiments the structural requirements for the action of pentacaine were analyzed. The strong inhibitory effects of pentacaine seem to be dependent on the molecule of the drug as a whole since its fragments alone or in combination did not produce a corresponding effect. The optimal side chain length of the alkylsubstituent seems to be $-OC_5H_{11}$. An analog with a shorter side chain had weaker inhibitory effects than pentacaine, and the local anaesthetics with longer alkyloxysubstituents tested did not have stronger effects. The position of the alkyloxysubstituent (meta- or para-) is not critical for the inhibitory effect on the adenylate cyclase activity (Fig. 6) although it substantially influences the local anaesthetic properties (Beneš, personal communication).

The inhibitory effect of pentacaine is not confined to the adenylate cyclase system in rat liver only. We could show inhibitory effects of this compound with other adenylate cyclase as well, namely from rat heart, rat adipose tissue and turkey erythrocyte ghosts (Hynie and Klenerová 1983; Hynie 1984; unpublished results). We also tested effects of tetracaine and pentacaine on ^{125}I-(−)-pindolol binding in turkey erythrocyte ghosts (Hynie and Klenerová 1983). Pentacaine was about four times more potent in inhibiting the radioligand binding to beta-adrenergic

receptors in turkey erythtocyte ghosts than tetracaine. It is interesting to note that, unlike pentacaine, tetracaine inhibition of the radioligand binding to beta-adrenergic receptors was not accompanied by a decrease in adenylate cyclase activity. This might be associated with an increase in membrane fluidity, resulting in an enhanced adenylate cyclase activity by a mechanism independent on the beta-adrenergic receptor stimulation.

It can be concluded that pentacaine and some of its analogs are very potent inhibitors of the adenylate cyclase system from various sources. Their main effect, although not the only one, seems to be the direct inhibition of the catalytic unit of adenylate cyclase. It seems to be promising to test these drugs as possible antagonists of excessive cyclic AMP production under pathological conditions characterized by increased adenylate cyclase activity, as occurring in cholera (Sharp and Hynie 1971).

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