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The Effects of Adrenergic Agonists on Intraocular Pressure and on Adenylyl Cyclase Activity of Ciliary Processes in Pigmented Rabbits

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Summary

The effects of β-adrenergic agonists isoprenaline, fenoterol and clenbuterol on the activity of adenylyl cyclase from ciliary processes and on intraocular pressure were examined in pigmented rabbits. Isoprenaline, fenoterol and clenbuterol stimulated adenylyl cyclase activity *in vitro*, but clenbuterol behaved as a partial agonist. Preincubation of ciliary processes with any of these three drugs led to the heterologous desensitization of adenylyl cyclase to the stimulatory effects of beta-adrenergic agonists or vasoactive intestinal peptide (VIP). This desensitization was dose-dependent and was expressed mainly as a decrease of the highest effects of stimulatory drugs. The exact mechanism of this phenomenon is not yet known. After topical administration, all three tested β-adrenergic agonists decreased intraocular pressure with approximately the same intensity. The relationship between ocular hypotensive effects of β-adrenergic agonists and their effects on adenylyl cyclase of ciliary processes is discussed. It is concluded that ocular hypotensive effects of adrenergic agonists and other drugs stimulating adenylyl cyclase cannot be explained simply by stimulation or desensitization of adenylyl cyclase of ciliary processes.

Key words
Adenylyl cyclase – Adrenergic agonists – Ciliary processes – Desensitization – Intraocular pressure

Introduction

Adenylyl cyclase (AC) of ciliary processes (CP) is thought to play a pivotal role in the regulation of aqueous humor production and in turn in the regulation of intraocular pressure (IOP). It is the stimulation of this enzyme in CP, which is presumed to cause the decrease of IOP (Sears and Mead 1983, Sears *et al.* 1984, Sears 1985).

The presence of β_2-adrenergic receptors has been proven in CP of the rabbit (Schmitt *et al.* 1984, Mittag and Tormay 1985, Elena *et al.* 1987). These receptors are positively coupled to AC, i.e. their stimulation by β-adrenergic agonists leads to the activation of AC (Waitzman and Woods 1971, Neufeld and Sears 1974, Nathanson 1980, Čepelík and Černohorský 1981, Mittag *et al.* 1985, Bausher *et al.* 1987). This conforms well, at the first glance, to the hypothesis of Sears' group mentioned above, since different β-adrenergic agonists have repeatedly been shown to exert a powerful ocular hypotensive effects (Langham and Diggs 1974, Potter and Rowland 1978, Rowland and Potter 1980, Potter *et al.* 1983, Woodward *et al.* 1986). However, while the association of cAMP formation and the reduction of intraocular pressure seemed to be firmly established, some inconsistencies exist which limit the general validity of this hypothesis (for overview see Wax 1992 or Wax and Barrett 1993). As a resolution of these inconsistencies, it has been suggested that it might be the desensitization of AC in CP which accounts for ocular hypotensive effects of adrenergic agonists (Mittag and Tormay 1981, Wax 1992, Wax and Barrett 1993).

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It seemed therefore worthwhile to examine the stimulation and desensitization of AC of CP *in vitro* by some selected β-adrenergic agonists, to study the effects of these drugs on IOP *in vivo* and to relate these effects mutually, as is being presented in this study carried out in the rabbit.

Material and Methods

Animals

Pigmented rabbits of the Chinchila strain (2.5–3.5 kg) were used throughout this study. All procedures involving animals conformed with the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23).

Intraocular pressure (IOP) measurement

IOP was measured using an applanation pneumatonograph (Digilab Inc., Cambridge, MA.) in conscious animals after topical anaesthesia of the cornea with the drop of 0.25 % tetracaine. Drugs tested for the effects on IOP were dissolved in physiological saline (w/v) and administered topically in 50 μl volume into the conjunctival sac of one eye. The volume of 50 μl of physiological saline was applied into the contralateral conjunctival sac. IOP was measured immediately before application of the appropriate solution and subsequently in intervals of one or two hours for six hours. A group of rabbits whose one eye was treated with physiological saline and the contralateral eye was left without any treatment served as an additional control. The effects of treatment are expressed as a "net" effect of tested drugs (in mm Hg) and were calculated by subtracting the value of baseline IOP of the treated eye from the value of IOP of this eye in the appropriate time interval after the treatment. Only the effects in treated eyes are presented in the figures.

Adenylyl cyclase assay

Homogenates of ciliary processes were prepared as described previously (Čepelík and Hynie 1990). The protein content in homogenates was estimated with Folin-phenol reagent (Lowry *et al.* 1951). Adenylyl cyclase assay was performed using ^{32}P-alpha-ATP as the substrate. Radioactive cyclic AMP formed was separated using aluminum column chromatography preceded by Dowex 50 purification.

Preincubation of ciliary processes

The rabbits was sacrificed by an intravenous overdose of pentobarbital and eyes were immediately removed and stored in physiological saline in a refrigerator until the preparation of ciliary processes was performed.

Ciliary processes were excised from the appropriate number of eyes and collected together on ice in a drop of preincubation medium, i.e. Krebs-Henseleit buffer (pH = 7.4) containing (mmol/l): NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25, which was equilibrated with a gas mixture of 95 % O_2 plus 5 % CO_2. After careful mixing, ciliary processes were divided into approximately equal portions (about 10 mg of tissue) for preincubation.

Preincubation of portions of CP was performed in plastic 10 ml tubes in a water bath at 37 °C under ambient air condition and with occasional shaking of the samples in the following manner: each sample was firstly incubated for 5 min in 1 ml of the preincubation medium. Then 10 μl aliquots of concentrated aqueous solutions of the tested drugs were added to the tissue samples in order to obtain the desired final concentrations of drugs. Control samples received 10 μl of Krebs-Henseleit buffer. All samples were further incubated for 20 min. At the end of preincubation, 5 ml of warm (37 °C) preincubation medium was repeatedly added to each sample to dispose of the stimulating agents. After a 30 s time period, the fluid was aspirated, and the washing procedure was repeated four times, but in the last two washes the homogenizing medium (containing in mmol/l: $MgCl_2$, 12.5; EGTA, 1.25; Tris-HCl buffer, 75; pH = 7.5) was used. After the last wash, the tissue samples were homogenized in 0.6 ml of the homogenizing medium using an electrically driven tissue disintegrator and these "crude" homogenates were used for adenylyl cyclase assay as described above.

Drugs

Isoprenaline (l-isoproterenol hydrochloride), fenoterol hydrobromide, clenbuterol hydrochloride, propranolol hydrochloride, forskolin and vasoactive intestinal peptide (VIP) were from Sigma Chemical Co. (St. Louis, Mo).

^{32}P-ATP was prepared in our laboratory by the procedure of Symons (1977).

All other chemicals were commercial preparations and were used without further purification.

Statistics

Data are presented as mean values ± S.E.M. Statistical significance was tested using Student's t-test. The accepted level of significance for all results was $P < 0.05$.

Results

Effects on the intraocular pressure

In treated eyes, all three tested β-adrenergic agonists, i.e. isoprenaline, fenoterol and clenbuterol, elicited a dose-dependent decrease of intraocular

pressure (IOP) with approximately the same intensity of the peak effect, but with differences in the time of their maximal effect, duration of their action (Fig. 1) and in the onset of their action (data not shown). Moreover, the maximal ocular hypotensive effect could already be elicited by 0.1% concentration of isoprenaline but only by 1% concentrations of fenoterol or clenbuterol. In contralateral untreated eyes, only clenbuterol elicited a clear-cut decrease of IOP (not shown).

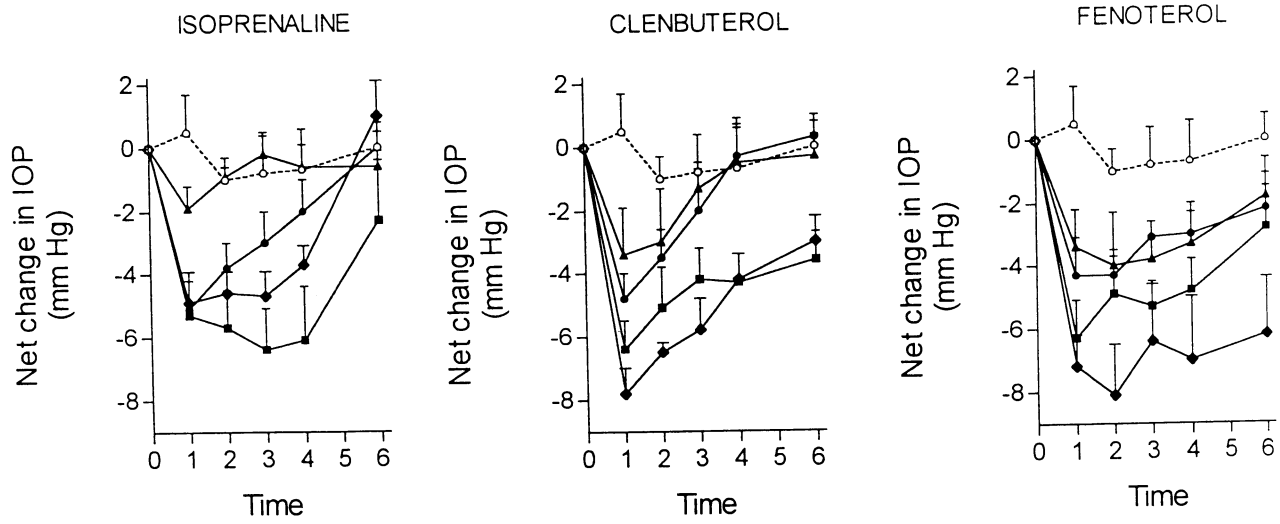

Fig. 1. *Effects of isoprenaline, clenbuterol and fenoterol on IOP in treated eyes (n=6). Axis x: Time in hours after the application of agonists. Net change in IOP was calculated by subtraction of baseline IOP (estimated at zero time) from IOP of tested agonist concentration at appropriate time. Baseline IOPs ranged from 22 to 24 mm Hg and did not differ significantly. All agonists were applied topically in following percentage concentrations: 0.0 (control animals, empty circles); 0.001 (triangles); 0.01 (filled circles); 0.1 (squares); 1.0 (diamonds).*

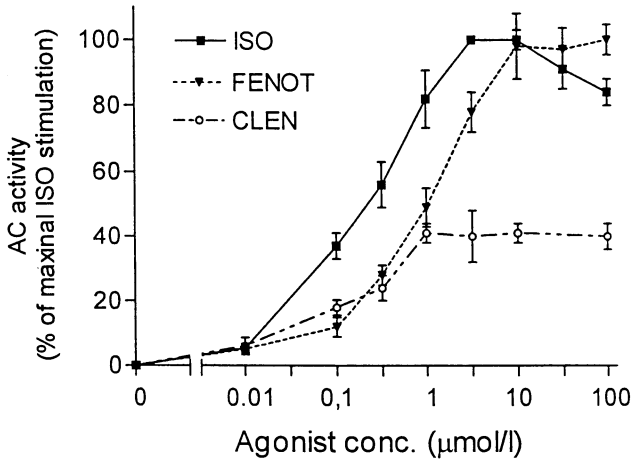

Fig. 2. *Stimulation of adenylyl cyclase activity by isoprenaline (squares), clenbuterol (empty circles) and fenoterol (triangles) (n=5). Net adenylyl cyclase activity in the presence of isoprenaline (3.16 µmol/l) was taken as 100 %. EC_{50} (in µmol/l) of particular agonists were following: isoprenaline 0.33±0.13; clenbuterol 0.23±0.22; fenoterol 1.0±0.24. EC_{50} of fenoterol was significantly different ($P<0.05$) from EC_{50} of isoprenaline or clenbuterol..*

Effects on the activity of adenylyl cyclase (AC) from ciliary processes (CP)

Isoprenaline, fenoterol and clenbuterol stimulated *in vitro* AC activity from CP (Fig. 2). However, remarkable differences in affinities and efficacies of these drugs to this receptor-adenylyl cyclase complex were found. Thus, isoprenaline exhibited the greatest affinity and efficacy as well. Fenoterol had about the same efficacy as had isoprenaline, but had lesser affinity than isoprenaline. Clenbuterol had approximately the same affinity as isoprenaline, but had a substantially lower efficacy than isoprenaline or fenoterol. All three tested drugs exhibited maximal or nearly maximal stimulatory activity at a concentration of 10 µmol/l.

Effects of preincubation of ciliary processes with different drugs on the sensitivity of adenylyl cyclase to stimulatory drugs

Preincubation of ciliary processes with isoprenaline, fenoterol or clenbuterol (10 µmol/l) led to a clear-cut and approximately the same decrease of adenylyl cyclase stimulation by isoprenaline or vasoactive intestinal polypeptide (VIP), without any sign of a decrease of the forskolin stimulatory effect (Fig. 3).

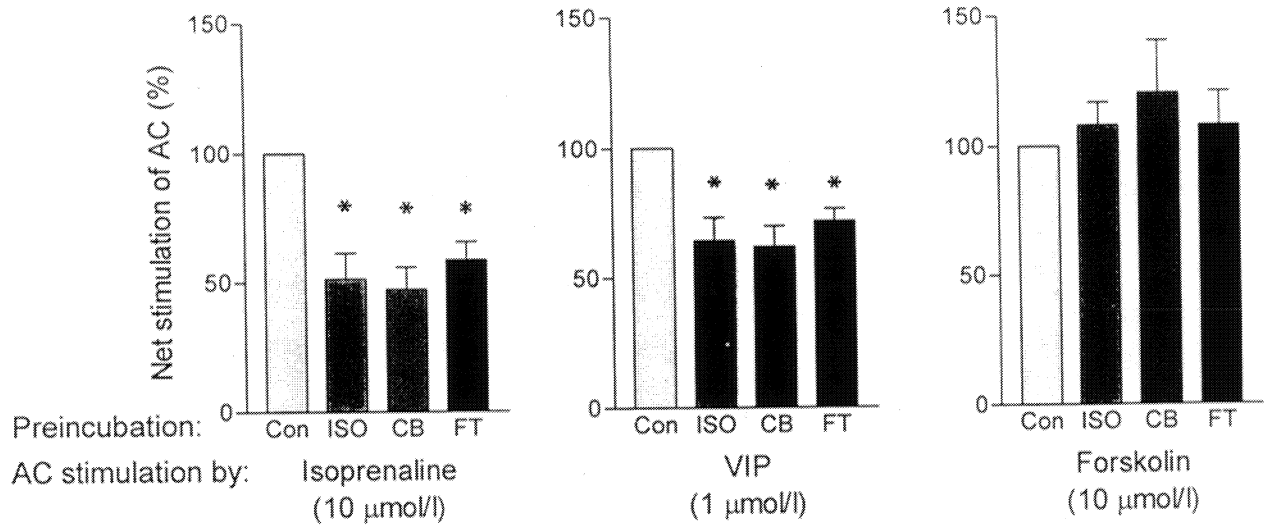

Fig. 3. *Effects of preincubation of ciliary processes with isoprenaline (ISO; 10 μmol/l), clenbuterol (CB; 10 μmol/l) or fenoterol (FT; 10 μmol/l) on the "net" stimulation of adenylyl cyclase by isoprenaline (10 μmol/l), VIP (1 μmol/l) or forskolin (10 μmol/l). "Net" stimulation of adenylyl cyclase was calculated by subtraction of basal activity from the adenylyl cyclase after stimulation by appropriate drug and its extent after control preincubation (Con; no drug added) of ciliary processes was taken as 100%. Asterisks indicate significant difference (P<0.05) against the activity after control preincubation as determined by paired t-test (n=6).*

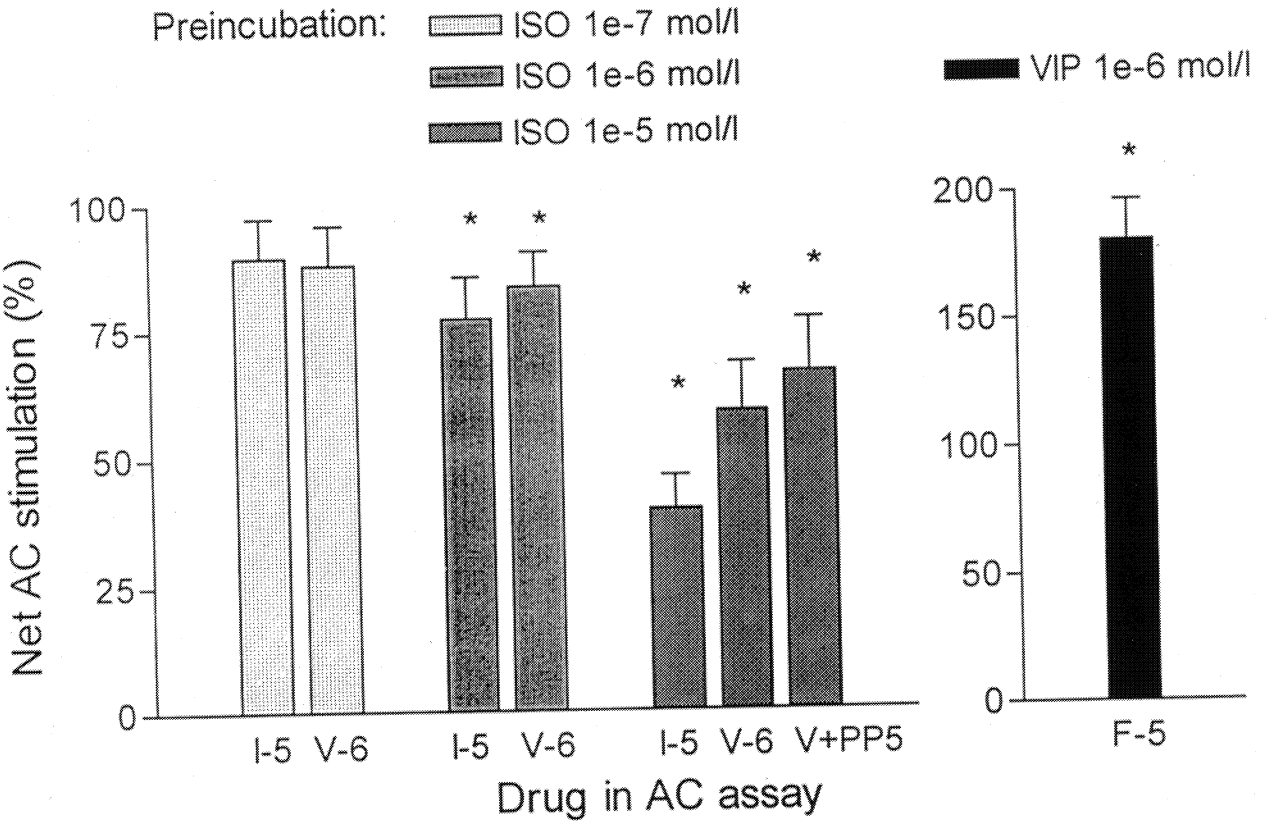

Fig. 4. *Effects of preincubation of ciliary processes with isoprenaline (0.1, 1 and 10 μmol/l,) (left side) or VIP (1 μmol/l) (right side) on the "net" stimulation of adenylyl cyclase by isoprenaline (I-5; 10 μmol/l), VIP (V-6; 1 μmol/l), VIP plus propranolol (PP5; 10 μmol/l) (left side) or forskolin (F-5; 10 μmol/l) (right side). "Net" stimulation of adenylyl cyclase was calculated by subtraction of basal activity from the adenylyl cyclase after stimulation by appropriate drug and its extent after control preincubation (no drug added) of ciliary processes was taken as 100 %. Asterisks indicate significant (P<0.05) reduction (left side) or increase (right side) of the activity against the activity after control preincubation as determined by paired t-test (n=6).*

Figure 4 shows that the decrease of stimulatory effects of isoprenaline or VIP as well as that elicited by preincubation of CP with isoprenaline at a concentration of 0.1 μmol/l was negligible and statistically non-significant. Preincubation with isoprenaline in a concentration of 1 μmol/l led to a clear-cut and statistically significant decrease of stimulatory effects of both drugs. Preincubation with isoprenaline at a concentration of 10 μmol/l led to a profound decrease of the stimulatory effects of isoprenaline and VIP, but with an apparently higher reduction of the stimulatory effect of isoprenaline. Figure 4 further shows that the decrease of VIP stimulatory effect by the preincubation of CP with isoprenaline (10 μmol/l) was not influenced by the presence of β-adrenergic antagonist propranolol (10 μmol/l) in the adenylyl cyclase assay. Furthermore, it is shown that Preincubation of CP with VIP (1 μmol/l) markedly enhanced adenylyl cyclase stimulation by forskolin (10 μmol/l).

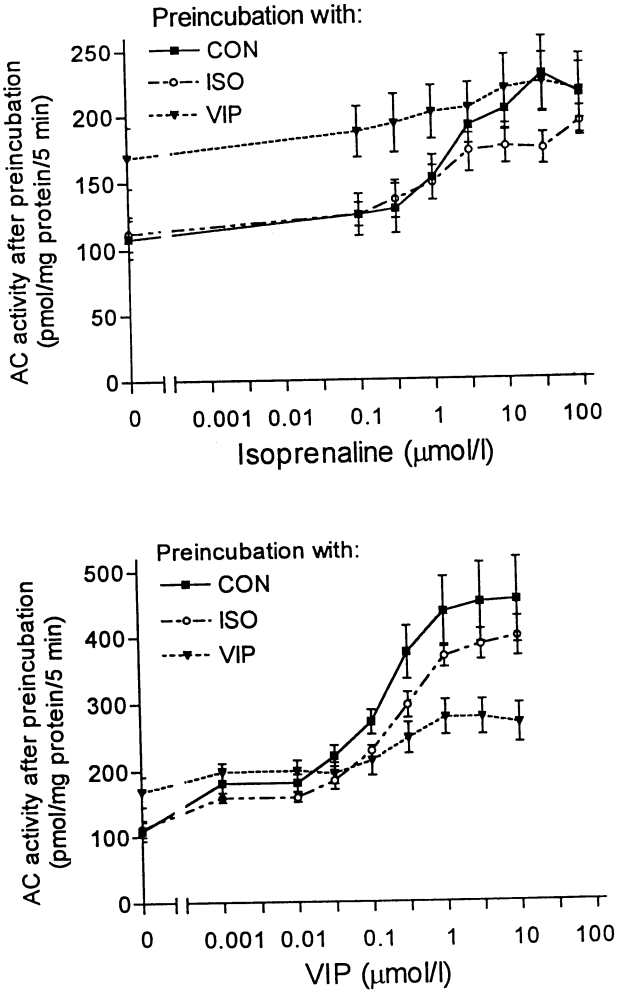

Fig. 5. *Effects of preincubation of ciliary processes with isoprenaline (10 μmol/l) or VIP (1 μmol/l) on the stimulation of adenylyl cyclase activity by isoprenaline or VIP (n = 4).*

Figure 5 shows that preincubation of CP with isoprenaline (10 μmol/l) did not influence the basal activity of adenylyl cyclase (Fig. 5), while preincubation of CP with VIP (1 μmol/l) led to a considerable increase of the basal AC activity. However, this increase did not exceed the stimulatory effect of the lowest VIP concentration used in the AC assay. Figure 5 also shows that preincubation of ciliary processes with isoprenaline or VIP led solely to a decrease of the stimulatory effects of both isoprenaline and VIP at highest concentrations without any apparent change in the affinities of any of these drugs.

Discussion

The primary aim of this study was to compare the effects of selected β-adrenergic agonists, i.e. isoprenaline, fenoterol and clenbuterol, on intraocular pressure (IOP) and on the activity of adenylyl cyclase (AC) from ciliary processes (CP) of the rabbit. Furthermore, the effects of some other drugs on the desensitization of the receptor-adenylyl cyclase complex from CP were also studied.

As far as the stimulatory effects of tested β-adrenergic agonists on the activity of AC from CP are concerned, the presented data show distinct differences in the affinity and maximal activity of isoprenaline, fenoterol and clenbuterol. Isoprenaline and clenbuterol stimulated AC activity of CP with approximately the same affinity, which was higher than the affinity of fenoterol. Moreover, isoprenaline and fenoterol exhibited almost the same maximal activity, while clenbuterol exhibited substantially lower maximal activity, i.e. it behaved as a partial agonist.

As concerns the desensitization of AC from CP to the stimulatory effects of different drugs which was elicited by the preincubation of CP with both β-adrenergic agonists or VIP, the examination of this phenomenon provided several interesting aspects which are discussed in the following paragraphs.

The extent of desensitization probably depend rather on the full occupation of adrenergic receptors than on the level of AC stimulation, as is suggested by approximately the same extent of the desensitization elicited by the maximal stimulatory concentration of the full agonists isoprenaline and fenoterol or partial agonist clenbuterol. The negligible extent of desensitization elicited by isoprenaline at a concentration of 0.1 μmol/l, i.e. a concentration at which isoprenaline displayed a stimulatory effect on AC of CP corresponding to the stimulatory effect of clenbuterol at concentration a 10 μmol/l also agrees with this proposal. Finding that the desensitization of AC was expressed mainly as a decrease of effects of the highest concentrations of both isoprenaline or VIP without any clear-cut change in affinities of these drugs conforms well to the finding that the desensitization of AC from rabbit iris-ciliary body elicited by isoprenaline

in vitro or by epinephrine *in vivo* was accompanied by none or a small decrease in number of β-adrenergic receptors or their affinity for ligand (Wax and Barrett 1993, Mittag and Tormay 1981).

An increase of basal activity of AC seen after preincubation of ciliary processes with VIP and negligible increase of basal activity of AC seen after preincubation of CP with β-adrenergic agonists might explain finding that preincubation of ciliary processes with VIP led to the potentiation of AC stimulation by forskolin while preincubation of CP with β-adrenergic agonists did not influence the forskolin effect on AC activity. The mutual desensitization of AC by isoprenaline and VIP indicates that the desensitization of AC from CP evoked by β-adrenergic agonists or VIP is heterologous. The exact mechanism of the desensitization of AC from CP is presently unknown, but a possible underlying cause might be an alteration of the catalytic unit of AC as have been suggested by Wax and Barrett (1983).

Surprisingly, the ability of tested β-adrenergic agonists to lower IOP in rabbits did not correspond to their power to stimulate AC activity of CP at all. All tested drugs exhibited approximately the same maximal ocular hypotensive effect i.e. they all exhibited full agonism. The main differences in the ocular hypotensive effect of tested drugs were apparent in the effectiveness of their lowest used concentrations, in their effect on the contralateral eye and in the duration of their action. Two last differences might probably be ascribed to differences in basic physico-chemical properties of tested drugs and most probably to differences in their partition coefficients. This is indicated by the fact that clenbuterol, which has the highest lipid-solubility from all used drugs, had the most prominent effect in the contralateral eye and its ocular hypotensive effect was the shortest.

For the finding that isoprenaline at 0.1% concentration elicited more profound ocular hypotensive effect than at 1% concentration we cannot offer any acceptable explanation, but similar pattern of the dose-response relationship of ocular hypotensive effect of dl-isoprenaline in rabbits has been shown (Seidehamel *et al.* 1975).

Discrepancy in the effects of tested β-adrenergic agonists on AC from CP and on IOP discussed above seems to be quite surprising from the point of view of a rather generally accepted idea that it is the stimulation of AC of CP that leads to decrease of IOP (Sears and Mead 1983, Sears *et al.* 1984, Sears 1985). Accepting this idea, one might suppose that the effectiveness of several β-adrenergic agonists at stimulation of AC of CP will correspond to their effectiveness at decreasing IOP. The finding that drugs with partial agonistic activity on AC of CP exhibited full agonism when tested as ocular hypotensive agents indicates that a relationship between the stimulation of AC of CP elicited by various drugs and between their ocular hypotensive effects may not be so straightforward as the original idea implicated (Sears and Mead 1983, Sears *et al.* 1984, Sears 1985).

Comparison of desensitization of CP β-adrenergic receptor-adenylyl cyclase complex by tested β-adrenergic agonists and their ocular hypotensive effects seems to conform to the idea that the desensitization of AC from CP induced by β-adrenergic agonists leads to a decrease of the effect of endogenous sympathetic stimulation with a decrease of aqueous humor production and subsequent decrease of IOP (Mittag and Tormay 1981, Wax and Barrett 1993). However, we have shown that repeated topical administration of fenoterol (1 %) *in vivo* led to the desensitization of β-adrenergic receptor-adenylyl cyclase complex from CP similar to that shown in present study *in vitro*, but it was accompanied by the change of initial ocular hypotensive effect of fenoterol to ocular hypertensive effect (Čepelík and Hynie 1997).

There is, of course, also a possibility that the stimulation of AC of CP by a particular drug and its ocular hypotensive effect are two independent processes that are influenced by the drug coincidentally but without any causal relationship. Accordingly, ocular hypotensive effects of drugs activating AC of CP might be exerted by their another action, possibly even independent on the stimulation of AC of CP. From this point of view a possible effect of β-adrenergic agonists and cyclic AMP on aqueous humor outflow (Lorenzetti 1971, Neufeld *et al.* 1975, Green *et al.* 1981) should not be omitted from a consideration.

In summary, the present study shows that, in the rabbit, several β-adrenergic agonists stimulated the activity of AC of CP *in vitro* and decreased IOP *in vivo* without any apparent correlation of both these effects. Accordingly, our results could be an additional piece of indirect evidence suggesting that there need not be any direct and simple causal relationship between the stimulation of AC of CP by drugs and their ocular hypotensive effect (Wax 1992, Hynie and Čepelík 1993, Čepelík et al. 1997). Though the ocular hypotensive effects of β-adrenergic agonists seem to correlate better with their ability to desensitize β-adrenergic receptor-adenylyl cyclase complex of CP, additional data mentioned above suggest that even desensitization of AC from CP can hardly be the underlying mechanism of ocular hypotensive effects of β-adrenergic agonists. Thus, it seems clear that the definitive explanation of mechanism on molecular basis of ocular hypotensive effects of β-adrenergic drugs and other drugs known to stimulate AC of CP will require further study.

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