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THE FOUR STEREOISOMERS OF A HIGH POTENCY CONGENER OF ISOPROTERENOL

BIOLOGICAL ACTIVITY AND THE RELATIONSHIP BETWEEN THE NATIVE AND THE CHEMICALLY INSERTED ASYMMETRIC CARBON

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Abstract—The RR isomer of a *para*-trifluoromethyl anilide congener of isoproterenol (PTFMA) had an affinity eighty and one hundred times higher than (−)isoproterenol for the beta receptor of turkey erythrocytes and of S49 cells respectively. This affinity was also much higher than that of ±hydroxybenzyl isoproterenol (HBI) tested in the same experiments.

The chemically inserted asymmetric carbon seemed to be as important as the native asymmetric carbon of the catecholamines in determining the binding affinity. Thus the RS and SR isomers demonstrated similar affinities in the turkey erythrocyte membranes as well as in the S49 lysed cells. The RR isomer had the lowest K_{act} in activation of adenylate cyclase in both beta receptor systems. The three most potent PTFMA isomers showed a K_{act}/K_d ratio which was higher than that of (−)isoproterenol or (±)HBI. It is therefore possible that the large substituent on the amino group in PTFMA, which greatly increases the binding affinity, is not as efficient in receptor activation. Yet the RR isomer had a K_{act} considerably lower than that of (−)isoproterenol in both of the beta receptor systems. The type of beta receptor of the turkey erythrocyte could be distinguished from that of the S49 cells by comparing the relative order of affinities of the RS and SR isomers and also by comparing (±)HBI with (−)isoproterenol. A labeled RR isomer of PTFMA could become most useful as an agonist ligand for beta receptors because of its very high binding affinity for both $beta_1$ and $beta_2$ receptors.

The synthesis [1] and biological characterisation [2, 3] of a new series of catecholamine derivatives has been described.

This series is based upon derivatization of the nitrogen of norepinephrine with a *p*-trifluoromethyl anilide (PTFMA)§ as shown in Fig. 1.

We have recently reported the binding affinity and the adenylate cyclase activation of a mixture of the four stereoisomers of PTFMA [4]. The studies were conducted on the $beta_1$ adrenergic system of turkey erythrocyte membranes and on the $beta_2$ system of S49 lymphoma cells. The potency of the racemic compound was one to two orders of magnitude higher than that of isoproterenol depending on the systems studied.

It is shown in Fig. 1 that the chemical synthesis of PTFMA has introduced an asymmetric carbon, alkylating the amino group, in addition to the native asymmetric carbon present in all beta-hydroxylated catecholamines. The two asymmetric carbons result in four different stereoisomers, each of which has been synthesized recently.‖ The present study characterizes each isomer with respect to binding to the receptor and activation of the adenylate cyclase in turkey erythrocyte membranes and in S49 lysed cells. Surprisingly, it is found that the conformation of the additional asymmetric carbon introduced by synthesis is about as relevant to the interaction with the beta receptor as the conformation of the asymmetric carbon native to the beta hydroxylated catecholamines.

Hydroxybenzylisoproterenol (HBI) is known to have a higher affinity for $beta_2$ receptors than isoproterenol [5–7] while it has been stated that it is equipotent with isoproterenol on the $beta_1$ receptor of turkey erythrocytes [8]. However, data about this latter point have apparently not been presented.

§ Abbreviations used: beta receptor, beta adrenergic receptor; PTFMA, *p*-trifluoro methyl anilide derivative; the stereoisomers of PTFMA are referred to according to their structure, RR, RS, SR, SS, where the first letter designates the asymmetric carbon adjacent to the catechol ring, while the second letter designates the carbon alkylating the amine; HBI, hydroxy benzyl isoproterenol; [^{125}I]CYP, [^{125}I] labeled iodocyanopindolol.

‖ To be reported elsewhere.

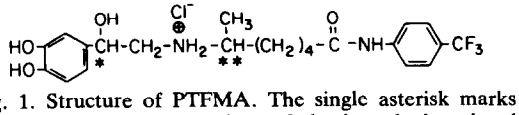

Fig. 1. Structure of PTFMA. The single asterisk marks the native asymmetric carbon of the beta hydroxylated catecholamine while the double asterisk marks the chemically inserted asymmetric carbon.

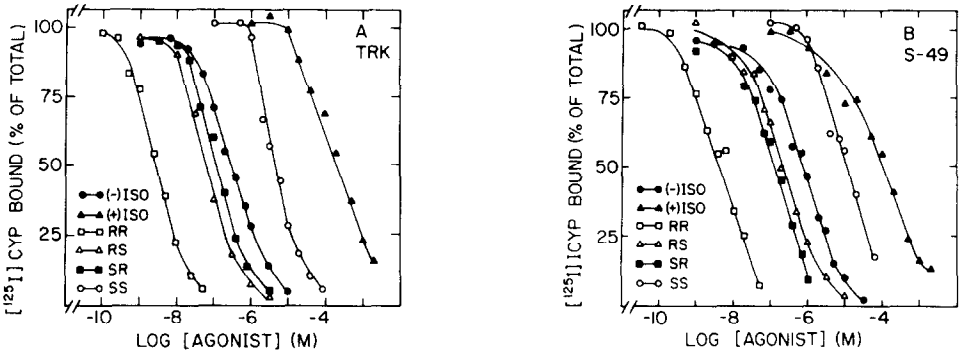

Fig. 2. Binding as a function of concentration of the four PTFMA isomers and isoproterenol (ISO) measured by competition with [^{125}I]ICYP. (A) Turkey erythrocyte membranes (TRK); (B) S49 lysed cells (S49).

Therefore HBI has been included in the present study to compare it with PTFMA and isoproterenol.

MATERIALS AND METHODS

The four stereoisomers of PTFMA, RR, RS, SR and SS were synthesized in Dr. Goodman's laboratory.* The same isomers were also kindly donated by Hoffman La Roche. Samples of (±)HBI were gifts from Dr P. B. Molinoff and from Dr R. J. Lefkowitz; isoproterenol was a product of Sigma. The catecholamines were dissolved in 10 μM HCl. Dilutions were prepared just prior to assay in a solution containing 1 μM HCl, 3 mM mercaptoethanol and 1 mM catechol. Other reagents were of highest purity available.

Membrane and lysed cell preparations. The procedure for turkey erythrocyte membranes was previously described [9]. Lysed S49 cells were prepared as follows. Cells from a fresh culture [5] were sedimented and washed in a solution containing (mM) NaCl 135, KCl 5, $MgCl_2$ 0.8, Tris buffer, pH 7.4, 20. The pellet obtained after centrifugation was suspended for lysis in a medium containing (mM) Tris buffer, pH 7.4, 10, mercaptoethanol 1, and $MgCl_2$ 2 to give a concentration of 2–4 × 10^7 cells/ml. This suspension was used for adenylate cyclase and for binding assays.

Ligand binding to the beta-receptor. Agonist binding and determination of the K_d were performed by competition with the antagonist [^{125}I]CYP [10] as previously described [11].

Adenylate cyclase activity. The reaction mixture in a volume of 0.12 ml contained (mM concentrations): 4-morpholinopropane-sulfonic acid buffer, pH 7.5, 50 [alpha-^{32}P]ATP 0.6 (about 30 cpm/pmole), GTP 0.001, cyclic AMP 1, $MgCl_2$ 6, mercaptoethanol 2, theophylline 0.2. EGTA 0.2, catechol 1, creatine phosphate 12, creatine kinase 9 units/ml. The catechol was added to decrease nonspecific binding and to aid in preservation of the catecholamines. The reaction was started by the addition of 50 μg membrane protein from turkey erythrocytes or 10^6 lysed S49 cells from the culture. Incubation was 10 min at 37°, during which cyclic AMP synthesis progressed linearly. The reaction was stopped by boiling and analyzed for [^{32}P]cyclic AMP according to Salomon *et al.* [12].

K_{act} values for the different catecholamines were calculated from Lineweaver–Burk plots.

Reproducibility and accuracy. All experiments were repeated at least twice, but most were performed several times. All assays were run in duplicate. The figures show typical experiments. Deviation from the mean was within ±6% and is therefore not indicated in the figures. The tables report the average K_d and K_{act}, with the SEM, calculated from all the repeats of experiments performed.

RESULTS

Binding of the PTFMA isomers to the beta-receptor of turkey erythrocyte membranes and S49 lysed cells

Figure 2 shows the competition of the above agonists with the labeled antagonist [^{125}I]CYP. For the turkey erythrocyte receptor (Fig. 2A) the order in decreasing affinity is RR > RS > SR > (−)isoproterenol > SS > (+)isoproterenol.

The concentration curve for RR is about two orders of magnitude to the left of (−)isoproterenol. Surprisingly, RS with the native asymmetric carbon in the configuration as in (−)isoproterenol, shows only a slightly higher affinity than SR. In sharp contrast, (+)isoproterenol runs about two orders of magnitude to the right of (−)isoproterenol. A similar, but not identical, order of affinities is displayed in Fig. 2B by the S49 lysed cells. The difference is that in the S49 system SR has even a slightly higher affinity than RS. It should be emphasized that RS and SR were compared several times, testing turkey erythrocyte membranes and S49 lysed cells in the same experiment, using the same dilutions of the agonists. We believe that the inversion of the order of affinities of RS versus SR, in going from turkey erythrocyte membranes to the S49 preparations, is real and is due to the difference between the respective receptors.

Figure 3 compares (±)HBI with (−)isoproterenol in binding to the beta-receptor. Clearly (−)isoproterenol demonstrates a higher affinity than (±)HBI for the turkey receptor (Fig. 3A), while

* To be reported elsewhere.

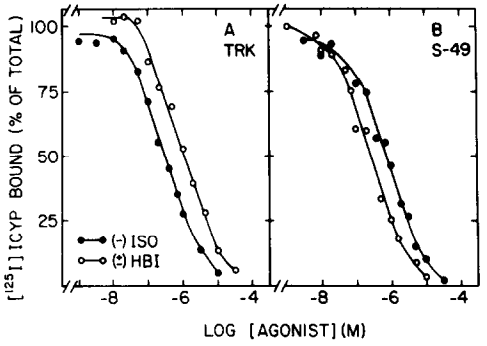

Fig. 3. Binding of HBI and isoproterenol. (A) and (B) as designated in Fig. 2.

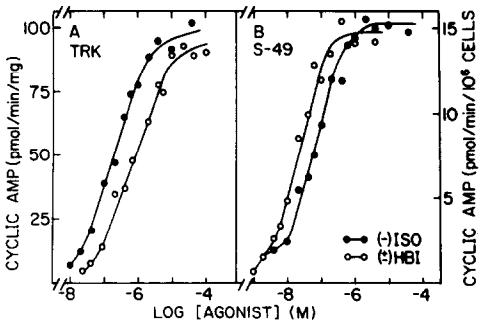

Fig. 5. Adenylate cyclase activation as a function of isoproterenol and HBI concentrations. (A) and (B) as designated in Fig. 2.

the reverse holds for the S49 receptor (Fig. 3B). Comparing Fig. 2 and 3 it is obvious that the RR isomer of PTFMA has a much higher affinity than (±)HBI for the beta receptor of both the turkey erythrocytes and the S49 lysed cells.

Figure 4 shows adenylate cyclase activation as a function of the concentration of the different catecholamines. Again, the highest potency is that of the RR isomer both for the turkey membranes (A) and for the S49 lysed cells (B). However, the second highest potency appears to be that of (−)isoproterenol for both beta receptor systems. As was shown in Fig. 2, with respect to binding to the receptor, (−)isoproterenol was only fourth in the order of potency. In the turkey system (Fig. 4A) RS displayed a slightly higher potency than SR and the latter failed to produce the same maximal activity as RS. In the S49 system (Fig. 4B) SR showed about the same, or a slightly higher potency than RS, both reaching a maximal activity about equal to that of RR and (−)isoproterenol. In the turkey system SR, SS and (+)isoproterenol failed to reach the maximal activity produced by RR and by (−)isoproterenol. The three former isomers also demonstrated some decline in activity at the highest concentration tested. Such a pattern is not evident in the S49 system, where all compounds except SS, reached about the same maximal activity.

Adenylate cyclase activation as a function of (±)HBI concentration in comparison to (−)isoproterenol is shown in Fig. 5. In the turkey system (−)isoproterenol shows a higher potency than (±)HBI while in the S49 system the reverse is true. Thus the relative potencies in adenylate cyclase activation (Fig. 5) parallel the relative affinities in binding to the receptors (Fig. 3). It should be noted that HBI was the racemic mixture since the (−)species was not available to us.

The data presented in Figs 2–5 and replicate experiments of the above served to calculate the K_d and K_{act} for the PTFMA stereoisomers, (±)HBI, (−) and (+)isoproterenol (Tables 1 and 2). In the turkey system (Table 1) RR has a binding affinity about eighty times higher than that of (−)isoproterenol. It is also evident that the RS and SR isomers have rather similar affinities, both higher than that of (−)isoproterenol. A rather surprising finding emerges when the ratio of K_{act}/K_d is calculated. While this ratio is about 1.5 for (−)isoproterenol as well as for (±)HBI it is greater than 8 for the three most active isomers of PTFMA. The pattern for the S49 beta receptor (Table 2) is quite similar to that noted above for the turkey receptor system. RR has a binding affinity more than one hundred times higher than that of (−)isoproterenol. The K_{act}/K_d ratio is about 0.3 for (−)isoproterenol and (±)HBI, while it is 3.8–5.7 for the three most active isomers of PTFMA. Thus the concentration of the PTFMA isomers producing half maximal adenylate cyclase activation is considerably higher

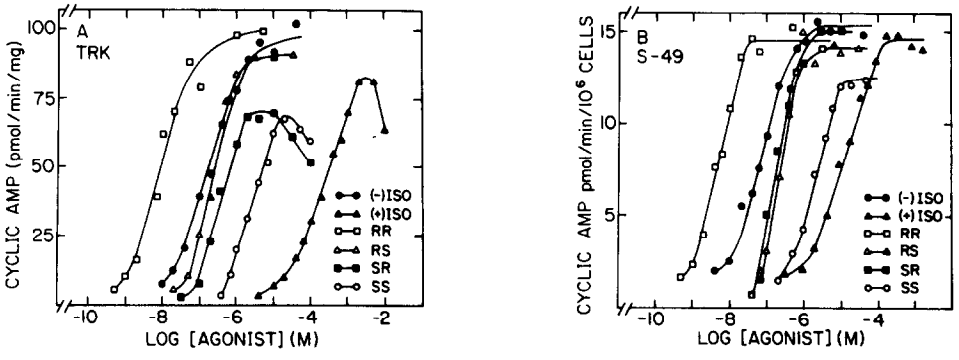

Fig. 4. Adenylate cyclase activation as a function of concentration of the four PTFMA isomers and isoproterenol. (A) and (B) as designated in Fig. 2.

Table 1. Turkey erythrocyte membranes: K_d and K_{act} values of the PTFMA isomers and other catecholamines

	Binding to the beta receptor		Adenylate cyclase activation		
Agonist	K_d (nM)	Affinity relative to (−)isoproterenol*	K_{act} (nM)	Potency relative to (−)isoproterenol†	K_{act}/K_d
(−)Isoproterenol	100 ± 5	1.0	160 ± 17	1.0	1.6
(±)HBI	400 ± 35	0.25	600 ± 27	0.27	1.5
PTFMA—RR	1.2 ± 0.2	83.0	10 ± 0.35	16.0	8.3
RS	30 ± 6	3.3	250 ± 35	0.64	8.3
SR	50 ± 0	2.0	650 ± 35	0.25	13.0
SS	3000 ± 800	0.03	7000 ± 1200	0.02	2.3
(+)Isoproterenol	80,000 ± 7000	0.001	120,000 ± 14,000	0.001	1.5

± Values show the SEM.
* K_d of (−)isoproterenol divided by K_d of agonist.
† K_{act} of (−)isoproterenol divided by K_{act} of agonist.

than the concentration producing half maximal binding to the receptor, while this is not the case for (−)isoproterenol and (±)HBI.

A number of experiments were conducted to find the cause for the difference in the K_{act}/K_d ratio between the PTFMA isomers on the one hand and (−)isoproterenol and (±)HBI on the other (not shown). Binding experiments were repeated in the presence of all adenylate cyclase assay reagents, including GTP. In the turkey erythrocyte system the K_d increased to the same extent for (−)isoproterenol and for RR, about threefold. Thus the difference in the K_{act}/K_d ratio between (−)isoproterenol and RR remained. The S49 system retested under the above conditions behaved similarly. Additional experiments tested the possibility that RR binding to the receptor does not reach equilibrium under the conditions of the binding assay which are satisfactory for (−)isoproterenol. However, increasing the incubation time in the binding assay from 30 to 90 min or changing the order of addition of membranes versus ligands had no effect on the results.

DISCUSSION

The studies presented clearly show that the RR isomer of PTFMA has the highest affinity for the beta receptor among the agonists tested. It also has the highest potency. In this respect there was no difference between the two beta receptor types tested; that of the turkey erythrocyte and that of the S49 cells.

An unexpected finding in this study is the important role of the second asymmetric carbon, which alkylates the amino group of the catecholamines (Fig. 1). It is perhaps not surprising that this asymmetric carbon may influence the configuration of the molecule and thus the binding to the receptor. However, one would not have predicted that the R configuration on the second asymmetric carbon could compensate for the "wrong" configuration, S, of the native asymmetric carbon. Thus the RS and the SR isomers showed similar binding affinities and also potencies in adenylate cyclase activation. In addition, the RS and SR isomers, as well as (±)HBI and (−)isoproterenol, can be used to distinguish

Table 2. S49 lysed cells: K_d and K_{act} values of the PTFMA isomers and other catecholamines

	Binding to the beta receptor		Adenylate cyclase activation		
Agonist	K_d (nM)	Affinity relative to (−)isoproterenol*	K_{act} (nM)	Potency relative to (−)isoproterenol†	K_{act}/K_d
(−)Isoproterenol	180 ± 40	1.0	60 ± 4.5	1.0	0.33
(±)HBI	70 ± 15	2.6	20 ± 0.3	3.0	0.29
PTFMA—RR	1.7 ± 0.3	106.0	9 ± 0.6	6.7	5.3
RS	70 ± 5	2.6	400 ± 0	0.15	5.7
SR	40 ± 7	4.5	150 ± 3	0.4	3.8
SS	3000 ± 100	0.06	1900 ± 100	0.03	0.63
(+)Isoproterenol	43,000 ± 5000	0.004	12,000 ± 1400	0.005	0.28

± Values show the SEM.
* K_d of (−)isoproterenol divided by K_d of agonist.
† K_{act} of (−)isoproterenol divided by K_{act} of agonist.

different beta receptor types. The order of relative affinities of these pairs of agonists for the turkey erythrocyte receptor was the opposite of that for the S49 receptor.

In passing it should be noted that the K_d values for (−)isoproterenol and (±)HBI, determined by competition with an ^{125}I labeled antagonist, differ quite markedly in different types of S49 preparations. Effects of guanyl nucleotides on the K_d do not seem a sufficient explanation for these differences (cf. present findings, [5, 7]). However, the emphasis of the present work is not on the absolute values for the different catecholamines but on their affinities and potencies relative to each other, compared in the same experiment.

In our previous work on racemic PTFMA the question was raised whether the extended structure beyond the amino group, which increases the affinity so dramatically, attaches to the receptor proper or to an associated lipid [4]. Although there is as yet no answer to this question, the present study does reveal an interesting difference between (−)isoproterenol and (±)HBI on the one hand and the PTFMA isomers on the other. For the former two catecholamines the K_d and the K_{act} are similar while for the PTFMA isomers, K_{act} is considerably higher than K_d.

It is therefore possible that the large substituent on the amino group of PTFMA, which is responsible for the impressive increase in binding affinity is somewhat less effective in conversion of the receptor to the active state. Yet, even with respect to adenylate cyclase activation, the RR isomer showed the highest potency. Therefore this isomer should become extremely useful in its labeled form, because it will permit the direct assay and characterization of beta receptor by agonist binding. Studies have already been performed using the agonist [^3H]-(±)HBI and these have revealed differences between agonist and antagonist binding [7]. However, this has only been possible with the $beta_2$ receptor which has a higher affinity for HBI than the $beta_1$ receptor. As demonstrated in the present study, the affinity of RR, even for the $beta_2$ receptor, is about forty times higher than that of (±)HBI.

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