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HIGH POTENCY CONGENERS OF ISOPROTERENOL

BINDING TO BETA-ADRENERGIC RECEPTORS, ACTIVATION OF ADENYLATE CYCLASE AND STIMULATION OF INTRACELLULAR CYCLIC AMP SYNTHESIS

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Abstract—The potency of a *p*-toluide derivative and a *p*-trifluoromethyl derivative of isoproterenol on the $beta_1$ receptor of turkey erythrocytes and on the $beta_2$ receptor of S49 lymphoma cells was measured in comparison with isoproterenol. Binding assays showed that the *p*-trifluoromethyl derivative possessed an affinity for the receptor, which was two hundred times higher than that of isoproterenol, in the case of turkey erythrocytes, and sixty times higher in the case of the S49 cells. By measuring the K_{act} of adenylate cyclase, the activity of the *p*-trifluoromethyl derivative was thirty to forty times higher than that of isoproterenol for the turkey erythrocyte membrane as well as for the S49 lysed cells. In stimulation of intracellular cyclic AMP accumulation, the K_{act} showed that the *p*-trifluoromethyl derivative was forty and twenty times more active than isoproterenol in turkey erythrocytes and in S49 cells, respectively. The *p*-toluide derivative gave similar results. The superior affinity of the above isoproterenol congeners for both $beta_1$ and $beta_2$ adrenergic receptors makes these compounds excellent candidates for use as labeled agonist ligands in studies of beta receptors. The possibility is discussed that the relatively large substituent on the amino group of the catecholamine congeners might perhaps bind to lipids associated with the receptor.

In the last few years a series of new isoproterenol congeners were synthesized [1], which when linked to peptides retained biological activity [2, 3]. Some of the low molecular weight aromatic derivatives in this series appeared considerably more potent than isoproterenol in stimulating cyclic AMP accumulation in intact S49 lymphoma cells [1]. Since high affinity agonists could be extremely useful for research on beta-adrenergic receptor systems it seemed of interest to characterize the action of these compounds. The *p*-trifluoro methyl anilide derivative (PTFMA)§ and the *p*-toluide derivative (PT) (Fig. 1) were therefore studied with respect to binding to the receptor, activation of adenylate cyclase and accumulation of cyclic AMP in intact cells, in turkey erythrocyte and in S49 lymphoma cell preparations. These two cell types were chosen because they represent well characterized beta-receptor systems belonging to two different receptor subclasses; the turkey erythrocytes being $beta_1$ [4] and the S49 cells being $beta_2$.

MATERIALS AND METHODS

The congeners of isoproterenol were synthesized in Dr Goodman's laboratory [1–3]; isoproterenol was a product of Sigma. The catecholamines, as hydrochlorides, 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 [5]. Lysed S49 cells were prepared as follows. Cells from a fresh culture [6] were sedimented and washed in a solution containing (mM) NaCl 135, KCl 5, $MgCl_2$ 0.8, Tris buffer, pH 7.4, 20. This solution will be referred to as salt medium. 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 final concentration of about 2×10^7 cells/ml. This suspension was used for adenylate cyclase and for binding assays.

Ligand binding to the beta-receptor. Agonist bind-

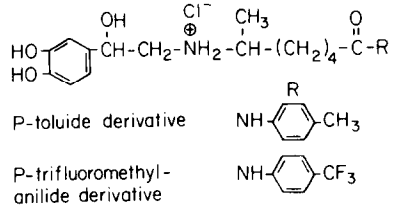

Fig. 1. Structure of the isoproterenol congeners, PT and PTFMA.

§ Abbreviations used: beta-receptor, beta-adrenergic receptor; PTFMA, *p*-trifluoro methyl anilide derivative; PT, paratoluide derivative.

ing and determination of the K_d were performed by competition with the antagonist ^{125}I-CYP [7] as previously described [8]. No GTP was added.

Adenylate cyclase activity. The reaction mixture in a volume of 0.12 ml contained (mM concentrations): 4-morpholinopropanesulfonic 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 non-specific 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.* [9].

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

Measurement of ^{3}H-cyclic AMP synthesis in intact turkey erythrocytes and S49 cells labeled by ^{3}H-adenine. Cells were incubated with 2-^{3}H-adenine to produce intracellular ^{3}H-ATP [10] as adapted in a previous study [11]. Fresh washed turkey erythrocytes were suspended (20% v/v) in Dulbecco modified Eagle medium containing 10% horse serum. ^{3}H-Adenine, 20 μCi/ml, was added and the suspension, 5 ml in a 50 ml Erlenmyer, was shaken under 95% O_2/5% CO_2 at 37° for 2 hr. The cells were washed and suspended in salt medium. The reaction mixture for ^{3}H-cyclic AMP synthesis contained in a final volume of 1 ml of salt medium, erythrocytes 2% v/v, 3-isobutyl-1-methylxanthine 0.5 mM, EDTA 0.2 mM, mercaptoethanol 1 mM, catechol 1 mM, with or without agonist. After incubation, 5 min at 37°, the amount of ^{3}H-cyclic AMP produced was determined as previously described [11]. The same procedure served also for study of ^{3}H-cyclic AMP synthesis in cultured S49 cells. The concentration of cells during labeling with ^{3}H-adenine was 3×10^7/ml and during ^{3}H-cyclic AMP synthesis about 1.5×10^7/ml.

Preliminary experiments showed that ^{3}H-cyclic AMP levels increased linearly, at least up to 10 min incubation. K_{act} values were calculated from Lineweaver–Burk plots.

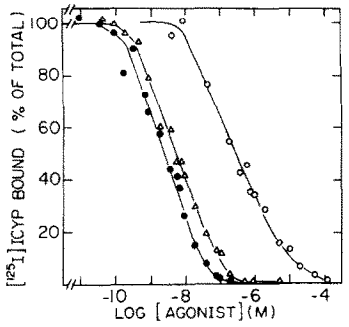

Fig. 2. Binding of PT and PTFMA to turkey erythrocyte membranes as a function of concentration measured by competition with ^{125}I-CYP. The procedure is described under Materials and Methods: ○——○, isoproterenol; △——△, PT; ●——●, PTFMA.

Reproducibility and accuracy. Isoproterenol served as a standard of comparison in each experiment. All experiments were repeated at least twice but most were performed several times. All assays were run in duplicate. Deviation from the mean was within ±6% and is therefore not indicated in the figures.

Reproducibility of results was improved by addition of 1–2 mM mercaptoethanol, as specified above. It had been reported that dithiothreitol reduces S—S bonds in the beta-adrenergic receptor resulting in a decrease of functional binding sites [12] or in their affinity [13]. A recent publication states that mercaptoethanol is ten to fifty times less efficient than dithiothreitol in reducing the beta-adrenergic receptor [14]. In the media and under the conditions specified in the present work, the low concentration of mercaptoethanol used had no effect on the number or affinity of beta-adrenergic receptors.

RESULTS

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

Figure 2 shows a representative experiment of

Table 1. K_d values for binding of isoproterenol and its new congeners to the beta-receptor on turkey erythrocyte membranes and on S49 cells

Agonist	K_d values obtained in individual experiments (nM)	Average K_{act} (nM)	Average affinity relative to isoproterenol*
Turkey erythrocyte membranes			
PT	4.0; 3.0; 1.5	2.8 ± 0.7	85
PTFMA	1.2; 1.5; 0.7	1.1 ± 0.2	215
Isoproterenol	400.0; 150.0; 160 ± 0	237 ± 82	—
S49 lysed cells			
PT	10.0; 20.0; 10.0	13.3 ± 3.3	24
PTFMA	3.6; 7.0; 5.0	5.2 ± 1.0	62
Isoproterenol	260.0; 500.0; 200.0	320 ± 92	—

* 1/average K_d of congener divided by 1/average K_d of isoproterenol

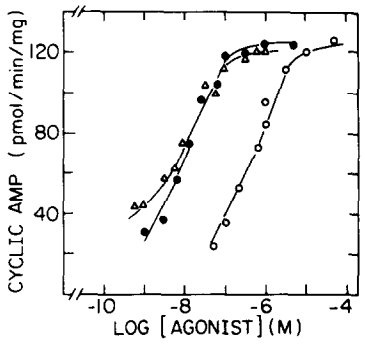

Fig. 3. Adenylate cyclase activation in turkey erythrocyte membranes as a function of the concentration of the isoproterenol congeners. The procedure is described under Materials and Methods: O——O, isoproterenol; Δ——Δ, PT; ●——●, PTFMA.

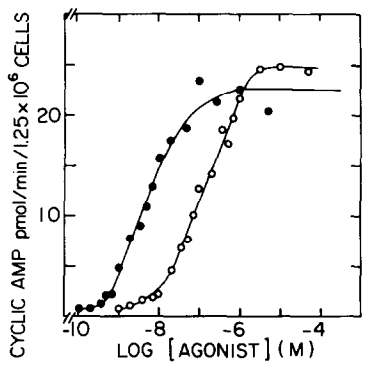

Fig. 4. Adenylate cyclase activation in lysed S49 cells as a function of PTFMA concentration: O——O, isoproterenol; ●——●, PTFMA.

competition between ^{125}I-CYP and the agonists for binding to the beta-receptor on turkey erythrocyte membranes. Smooth parallel curves are obtained for all three catecholamines. The much higher affinity of PTFMA and PT, relative to isoproterenol, is quite evident. The data in the experiment of Fig. 2 and repeats of this experiment served to calculate the K_d values for the new derivatives and these are given in Table 1. Evidently, the affinity of the congeners for the beta-receptor is roughly one hundred times higher than that of isoproterenol.

Binding to the beta-receptor on S49 lysed cells was studied in experiments identical to those shown in Fig. 2 for the turkey erythrocyte receptor. Table 1 also shows the K_d values for the S49 receptor. PTFMA demonstrates a K_d which is about one-fiftieth that of isoproterenol while PT has a K_d about twice as high as that of PTFMA. Thus, the affinity of the new cogeners for the receptor on S49 lysed cells is apparently slightly lower than their affinity for the turkey erythrocyte receptor.

Beta-receptor activation of adenylate cyclase by PT and PTFMA in turkey erythrocyte membranes and in S49 lysed cells

Having established the high affinity of the congeners for binding to the receptor their potency as agonists was investigated. Figure 3 shows a typical experiment of the relationship between the concentration of the isoproterenol congeners and adenylate cyclase activity in turkey erythrocyte membranes. Again, smooth parallel curves are obtained. The curves for PT and for PTFMA are essentially superimposable. The K_{act} values calculated from several experiments are given in Table 2.

For PTFMA the K_{act} values are somewhat higher than the K_d values (compare Tables 2 and 1). The same type of experiment served to derive the K_{act} value for PTFMA in activation of the beta-receptor coupled adenylate cyclase of S49 lysed cells (Fig. 4 and Table 2).

The average K_{act} value for PTFMA in S49 lysed cells is almost identical with that of the turkey erythrocyte membranes (Table 2).

In further studies stimulation of intracellular cyclic AMP synthesis by the isoproterenol congeners was investigated in intact turkey erythrocytes and in S49 cells. Figure 5 shows the rise in ^{3}H-cyclic AMP in turkey erythrocytes as a function of PTFMA concentration in comparison with isoproterenol concentration. The PTFMA curve is shown to be shifted to about 40-fold lower concentrations relative to the isoproterenol curve.

Table 2. K_{act} values of isoproterenol and its new congeners for adenylate cyclase activation in turkey erythrocyte membranes and in S49 lysed cells

Agonist	K_{act} values obtained in individual experiments (nM)	Average K_{act} (nM)	Average potency relative to isoproterenol*
Turkey erythrocyte membranes			
PT	3; 6	4.5 ± 1.5	67
PTFMA	4; 10	7.0 ± 3.0	43
Isoproterenol	200; 300 300; 400	300 ± 41	—
S49 lysed cells			
PTFMA	2; 2; 5; 5; 6; 8	4.7 ± 1.0	32
Isoproterenol	100; 130; 180; 200	153 ± 23	—

* 1/average K_{act} divided by 1/average K_{act} of isoproterenol.

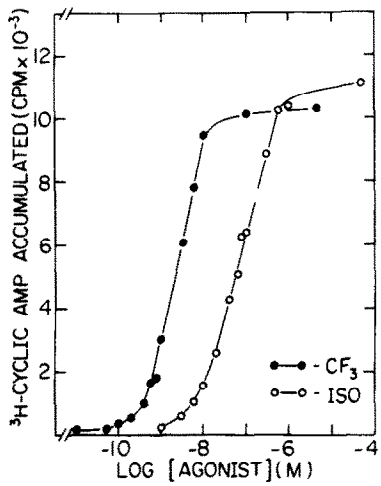

Fig. 5. Stimulation of intracellular ^{3}H-cyclic AMP synthesis in turkey erythrocytes as a function of the concentration of PTFMA and isoproterenol. The procedure is described under Materials and Methods: O——O, isoproterenol; ●——●, PTFMA.

The K_{act} values for stimulation of intracellular cyclic AMP synthesis are presented in Table 3. The average K_{act} of PTFMA for intracellular cyclic AMP synthesis and for adenylate cyclase activation are quite similar. However, the K_{act} of isoproterenol and of PTFMA for intracellular cyclic AMP synthesis in the turkey erythrocytes is lower than the K_{act} of adenylate cyclase activation in the membranes of the erythrocytes (Tables 2 and 3). This difference might be due to more efficient coupling of the receptor to the enzyme in the intact erythrocytes.

DISCUSSION

The present studies on the beta-receptor systems of turkey erythrocytes and of S49 cells clearly show that both PT and PTFMA have a potency which is much higher than that of isoproterenol. Assays of binding to the receptor, adenylate cyclase activation in membranes and stimulation of cyclic AMP synthesis in intact cells all support this conclusion.

Agonists with such high potency on $beta_1$ receptors, as demonstrated in the present study in the turkey erythrocyte systems have apparently not been reported previously.

PTFMA also appears to have the highest known binding affinity for $beta_2$ receptors. Hydroxybenzylisoproterenol was reported to have a ten to twenty times higher affinity than isoproterenol [15], while PTFMA has a sixty times higher affinity (Table 1). It is also of interest to note that the K_d of PT and PTFMA for the $beta_1$ receptor of the erythrocytes is about one-fifth that of the $beta_2$ receptor of the S49 cells. This difference in the K_d is probably due to the substituent linked to the amino group of those catecholamines since the K_d values of isoproterenol for the erythrocyte receptor and the S49 receptor are quite similar (Table 1).

Radioactive PT or PTFMA could be extremely useful in studies of agonist binding to beta-receptors. The disadvantages of studying agonist binding only indirectly, by displacement of an antagonist, have already been pointed out [16]. However, up to the present time no agonists with sufficiently high affinity were available for binding studies on $beta_1$ receptors.

An earlier survey of the potency of a large number of isoproterenol congeners was based on stimulation of cyclic AMP accumulation by S49 cells relative to isoproterenol [1]. The assay employed was different from the one used in the present study and indicated a higher potency of PTFMA relative to isoproterenol than observed in the present study. The reason for this discrepancy is not obvious. An assay on intact cells involves, of course, many unknown factors. It is quite possible, for instance, that higher resistance to oxidation of PTFMA relative to isoproterenol in a system which had not been stabilized by catechol and mercaptoethanol might explain the earlier relative values.

The higher potency of the new isoproterenol congeners is obviously due to the structure attached to the amino group of the catecholamine. Preparation of a large number of derivatives showed quite clearly that minor changes in this structure, although outside the catecholamine proper, dramatically affect the potency of the derivative [1, 3]. There are at least two alternative explanations for this observation: (i) The structure, beyond the catecholamine proper, also binds solely to the receptor protein; (ii) the extended part binds to specific lipids with which the

Table 3. K_{act} values of PTFMA and isoproterenol for stimulation of intracellular ^{3}H-cyclic AMP synthesis in turkey erythrocytes and S49 cells

Agonist	K_{act} values obtained in individual experiments (nM)	Average K_{act} (nM)	Average potency relative to isoproterenol*
Turkey erythrocytes			
PTFMA	1; 2; 2	1.7 ± 0.3	39
Isoproterenol	70; 60; 70	67 ± 3.3	—
S49 cells			
PTFMA	2; 4; 5	3.7 ± 0.9	18
Isoproterenol	40; 60; 100	67 ± 18	—

* See footnote to Table 2.

receptor interacts. It might soon become possible to investigate this important question. Thoroughly delipidated crude fractions of the beta-receptor and of the GTP binding protein have been reconstituted with specific lipids [8, 17]. If the K_d and K_{act} for PTFMA in the native membrane are different from those in the system relipidated with various defined lipids, while the K_d and K_{act} for isoproterenol are found not to change appreciably, it would suggest that the specific lipids play a role in the binding of the large substituent attached to the amino group of the catecholamine.

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