ELSEVIER

European Journal of Pharmacology
Molecular Pharmacology Section 267 (1994) 63–69

ejp
molecular pharmacology

Thermodynamic analysis of isoproterenol binding to β-adrenoceptors in rat lung membranes

Norbert Fraeyman *, Ariane Bazan, Patrick Vanscheeuwijck

Heymans Institute of Pharmacology, University of Gent, Medical School, De Pintelaan 185, B-9000 Gent, Belgium

(Received 15 September 1993; accepted 16 November 1993)

Abstract

The thermodynamic properties of the binding of the β-adrenoceptor agonist isoproterenol and of the antagonist propranolol to β-adrenoceptors of rat lung were investigated. We found that in our experimental conditions, the high- and low-affinity binding sites for the agonist displayed different properties: the binding to the high-affinity binding site was entropy-driven with a small increase in enthalpy, while agonist binding to the low-affinity binding site was enthalpy-driven. Binding of isoproterenol in the presence of GTP or its non-hydrolyzable analogue GppNHp, and the binding of propranolol were enthalpy-driven with a small increase in entropy.

Key words: Thermodynamics; β-Adrenoceptor agonist interaction; Lung (rat)

1. Introduction

The interaction of agonists to β-adrenoceptors is characterized by the presence of both high- and low-affinity binding sites. Thus, in competition binding experiments using the agonist isoproterenol as competitor and the radioligand [^{125}I]iodocyanopindolol which is an antagonist with no selectivity for the agonist binding sites, biphasic curves are usually obtained. Addition of guanosine trisphosphate (GTP) or of a non-hydrolysable analogue such as guanilylimido bisphosphate (GppNHp) converts high-affinity binding sites into low-affinity binding sites equivalent to the low-affinity binding sites detectable in the absence of guanine nucleotides (Helmreich and Pfeuffer, 1985; Levitzki, 1988). Two models have been introduced: the 'two-state' model in which a dynamic equilibrium between both types of agonist binding site is proposed, and the 'two-site' model, in which high- and low-affinity binding sites are considered to bind the agonist independently (Contreras et al., 1986). It is possible that the distinction between these two models might be subtle, as calculations related to both models yield very similar results.

In a recent series of experiments we became interested in the effect of aging on the thermodynamic properties of the binding of antagonists and agonists to β-adrenoceptors in rat lung. The results of the binding of the antagonist iodocyanopindolol have been published (Fraeyman and Vanscheeuwijck, 1991). We subsequently started the study of the interaction of isoproterenol with lung membranes of rats of different age. During these experiments, we became aware of fundamental differences between our results and what is known on the thermodynamics of the β-adrenoceptor–isoproterenol interaction. We therefore investigated in detail the thermodynamic characteristics of this interaction in lung from rats of 2 to 3 months of age.

In contrast to the results of Contreras et al. (1986), we observed that agonist binding to the high-affinity binding site is entropy-driven, with an increase in enthalpy, while agonist binding to the low-affinity binding site is, as the binding of the antagonist propranolol, enthalpy-driven accompanied by small increases in entropy.

* Corresponding author. Tel: 32-9-240.33.84; Fax: 32-9-240.49.88.

SSDI 0922-4106(93)E0192-M

2. Materials and methods

2.1. Materials

(−)-[^{125}I]Iodocyanopindolol was obtained from Amersham (Amersham, UK). (−)-Propranolol and (−)-isoproterenol were obtained from Sigma (Poole, UK). Guanosine trisphosphate and guanylylimido bisphosphate were purchased from Boehringer-Mannheim (Germany). All other reagents were of the highest purity commercially available.

2.2. Animals

Male Wistar rats aged 2 to 3 months were obtained from the Center for Experimental Animals of the Katholieke Universiteit of Leuven (Belgium). They were housed individually and had free access to food and acidified water; before the experiment, they were fasted overnight.

2.3. Tissue preparation

Rat lung membrane preparations were obtained as described (Vanscheeuwijck et al., 1989b). Briefly, lungs were homogenized in sucrose/Tris/EGTA buffer (250/50/2 mM, pH 7.4) using an Ultra-turrax homogenizer, three times for 15 s at a maximal speed with 1-min intervals. The homogenate was centrifuged at low speed (2000 × g for 10 min), subsequently at high speed (33,000 × g for 20 min) and washed three more times. The final crude membrane preparation was resuspended in Tris/$MgCl_2$/EGTA buffer (50:20:2 mM, pH 7.4).

In a separate series of experiments, the lung of each animal was devided in two parts; one portion was homogenized as above (i.e., in $MgCl_2$-free homogenization buffer). The second portion was homogenized in $MgCl_2$-containing buffer (Sucrose/Tris/$MgCl_2$/EGTA 250:50:20:2 mM, pH 7.4). Both homogenates were centrifuged and washed as described above in the corresponding buffers. The final pellet derived from the first portion was resuspended in $MgCl_2$-free buffer (Tris/EGTA 50:2 mM, pH 7.4), the pellet of the second portion was resuspended in the $MgCl_2$-containing buffer as mentioned above. In all cases, crude membrane preparations were used immediately for isoproterenol competition binding experiments (see further).

2.4. Binding experiments

Competition binding was done as described before (Vanscheeuwijck et al., 1989b), using (−)-isoproterenol (10^{-11} to 10^{-4} M) without or with GppNHp (10^{-4} M) or (−)-propranolol (10^{-11} to 10^{-5} M) as competitors, and radioactive iodocyanopindolol as ligand; approximately 5 μg of membrane protein was used. In competition and saturation binding experiments, incubation time was dependent on the temperature. In preliminary experiments (results not shown and Fraeyman and Vanscheeuwijck, 1991) optimal incubation time to assure complete equilibrium was determined: for 60 min when the incubation was at 37°C or at 30°C, for 3 h at 20°C and for 6 h at 4°C. Saturation binding experiments were done with 9 concentrations of [^{125}I]iodocyanopindolol ranging from 2 to 300 pM; non-specific binding was in preliminary experiments determined using 1 μM propranolol or 100 μM isoproterenol at every ligand concentration and found to be linear; in all further binding assays, non-specific binding was determined at the highest ligand concentration only and was always less than 20% of total binding. All incubations were done in microtiter plates and separation of free ligand from bound ligand was achieved using an automatic cell harvesting system (Vanscheeuwijck and Fraeyman, 1989a).

2.5. Calculations

Calculation of the parameters of the competition binding curves was done using the GraphPAD program (Motulsky, 1987). Each curve was analyzed according to a one and a two site model. The partial F-test was used to decide what model was most adequate (Snedecor and Cochran, 1973). K_i values were calculated according to Cheng and Prusoff (1973); at each temperature, K_d values were determined from saturation binding experiments and linear Scatchard analysis (Fraeyman and Vanscheeuwijck, 1991). When the two site model was accepted, the percentage of β-adrenoceptors in both configurations was given by the program; total binding was set as 100%. The standard

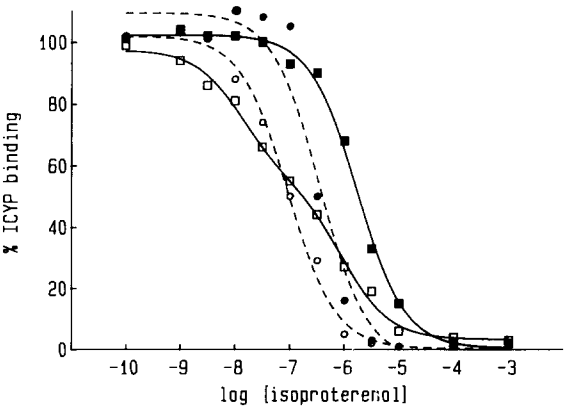

Fig. 1. Representative result of the competition binding of isoproterenol to a crude lung membrane preparation of the rat. Only the curves of binding at 10° and at 37°C are shown. Binding at 10°C (○) and at 37°C (□) was measured after 6 and 1 h, respectively. Empty symbols: without GppNHp filled symbols: with GppNHp.

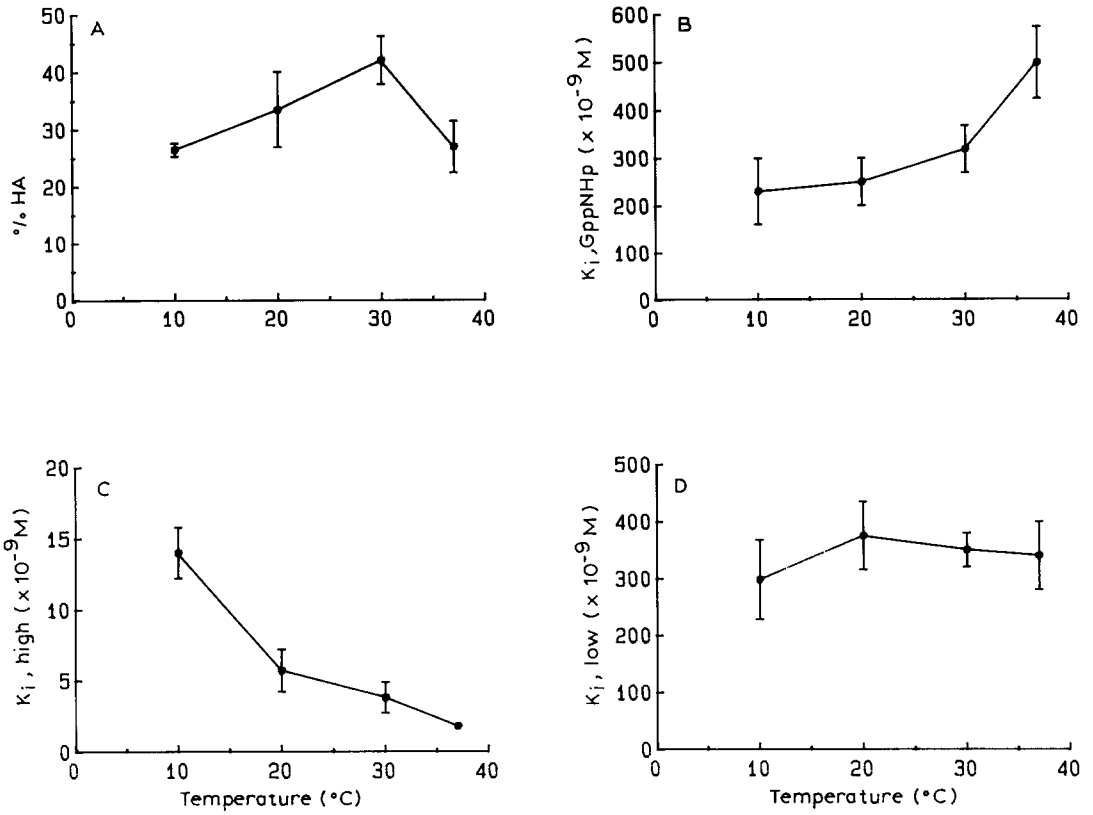

Fig. 2. Effect of temperature on the percentage of total binding present as high-affinity binding site (Fig. 2A, %HA), on the K_i value for high (Fig. 2B, $K_{i,high}$), GppNHp-induced (Fig. 2C, $K_{i,GPPNHP}$) and low (Fig. 2D, $K_{i,low}$) affinity binding site of isoproterenol determined in competition binding experiments. Each point of the curves is the mean of at least four independent determinations; means ± SEM are shown.

value for free energy was calculated from the dependency of the K_d values upon the temperature (Fraeyman and Vanscheeuwijck, 1991) as $\Delta G = RT \ln K_d$ by interpolation of the temperature-K_d value curve at 25°C. The enthalpy difference ($\Delta H°$) was derived from the Van 't Hoff plot; the entropy difference ($\Delta S°$) was calculated using the formula $\Delta G° = \Delta H° - T\Delta S°$. Statistical analysis was done using one-way analysis of variance when appropriate and when indicated; statistical significance was accepted at the $P < 0.05$ level.

3. Results

In Fig. 1 the results of a representative experiment of the influence of temperature on competition binding

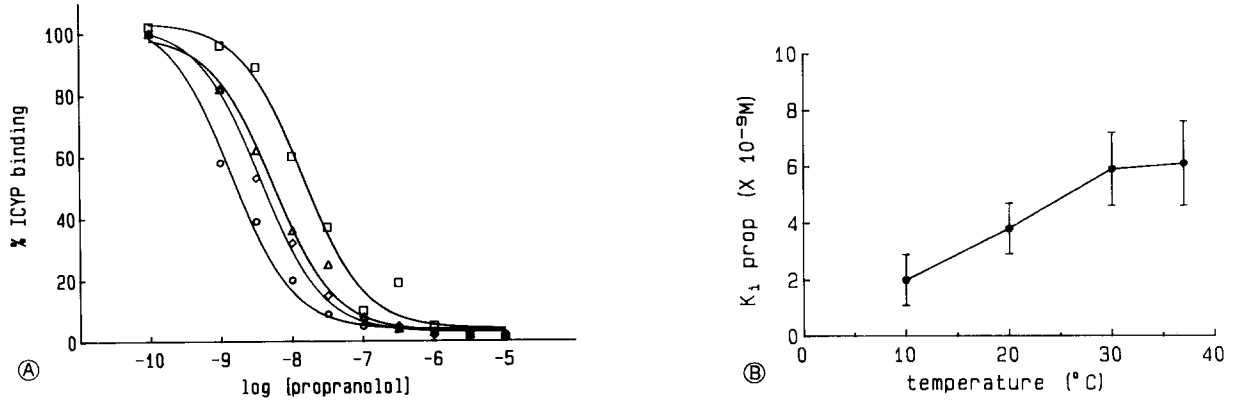

Fig. 3. Representative result of the competition binding of propranolol (Fig. 3A) to a crude lung membrane preparation of the rat. Binding was at 10°C for 6 h (○), at 20°C for 3 h (◇) at 30°C (△) and at 37°C (□) for 1 h. Fig. 3B: Effect of temperature on the K_i value for propranolol (K_i, prop) in competition binding experiments. Each point is the mean of at least 4 independent determininations; means ± SEM are shown.

with isoproterenol, in the absence and the presence of GppNHp, are shown; average values for the calculated inhibition constants (K_i values for the high- and low-affinity binding sites) and the percentage high-affinity binding sites as function of the temperature are depicted in Fig. 2. Changing the incubation temperature affects the K_i value of the high- and low-affinity binding sites differently: the equilibrium inhibition constant of the high-affinity binding site for isoproterenol (Fig. 2B) was decreased 4 to 5 fold, while that of the low-affinity binding site (Fig. 2D) was slightly (1.5 fold) increased upon increasing the temperature (both $P < 0.05$, one-way ANOVA). When GppNHp is added to the incubation mixture, only the low-affinity binding is detectable (Fig. 1). Increasing the temperature has a marked effect on the affinity of this GppNHp-sensitive binding site for isoproterenol: a highly significant decrease in affinity was noted ($P < 0.001$, one way ANOVA; Fig. 2C). The percentage of binding sites with high affinity for the agonist increased from 23.1% at 10°C to 42.0% at 30°C ($P < 0.05$, one-way ANOVA); increasing the temperature further to 37°C caused a drop to 26.9% (Fig. 2A).

The effect of changing the temperature on the binding of propranolol in competition binding experiments is summarized in Fig. 3A. In all cases, the one-site model explained the results. Increasing temperature is accompanied by a significant 2.5 fold increase in the equilibrium inhibition constant of the binding (Fig. 3B, $P < 0.05$, one-way ANOVA).

The results of the transformation of the binding data into Van 't Hoff plots is shown in Fig. 4; quantitative data are summarized in Table 1. For the high-affinity binding site, a positive enthalpy change which was accompanied by a large increase in entropy was calculated. For the low-affinity binding site, for the GppNHp-sensitive binding site and for the binding of propranolol, negative enthalpy changes accompanied by small increases in entropy were noted.

Table 1
Average values for standard free energy, enthalpy and entropy of the binding of isoproterenol and propranolol to a crude membrane preparation of rat lung

	$\Delta G°$ kcal/mol	$\Delta H°$ kcal/mol	$\Delta S°$ cal/mol-deg
Isoproterenol			
High affinity	−10.9	10.8	72.9
Low affinity	−8.6	−4.1	15.3
GppNHp	−8.6	−4.6	13.1
Propranolol	−11.8	−8.5	11.1

High affinity: high-affinity binding site for isoproterenol in the absence of GppNHp. Low affinity: low-affinity binding site for isoproterenol in the absence of GppNHp GppNHp: binding site for isoproterenol in the presence of GppNHp Propranolol: binding site for propranol in the absence of GppNHp.

The results of the separate series of competition binding experiments with the $MgCl_2$-containing and $MgCl_2$-deprived samples are shown in Fig. 5. Calculation of the thermodynamic parameters of the $MgCl_2$-

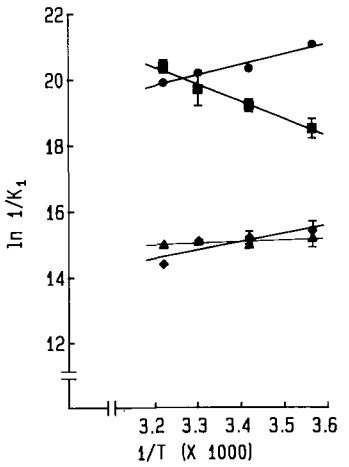

Fig. 4. Van 't Hoff plot of the K_i values derived from the competition binding experiments presented in Figs. 1 and 3. ●: propranolol; ■: high-affinity binding site and ◆: low affinity binding site for isoproterenol; ▲: GppNHp-sensitive binding site for isoproterenol.

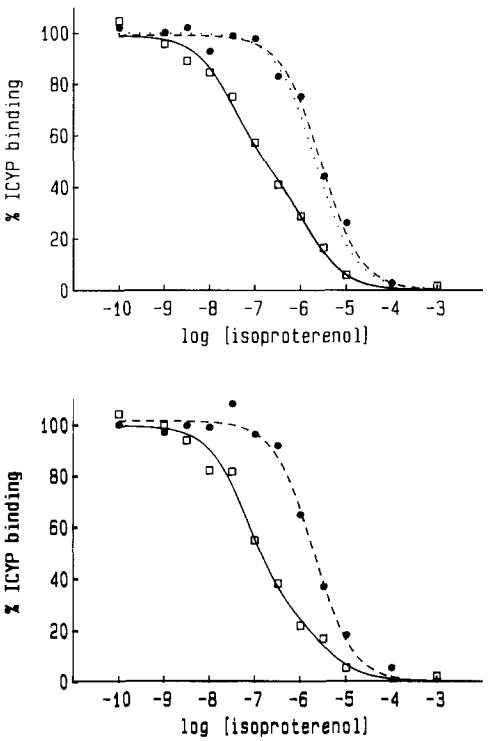

Fig. 5. Representative result of the competition binding of isoproterenol to a crude lung membrane preparation of the rat and the effect of $MgCl_2$ hereupon. Only the curves of agonist binding at 37°C (upper panel; 1 h of incubation) and at 10°C (lower panel; 6 h of incubation) are shown. Empty symbols: without GppNHp, $MgCl_2$-containing medium; full symbols: without GppNHp, $MgCl_2$-deprived medium; dotted line (for the experiment at 37°C only): with GppNHp in $MgCl_2$-containing medium (only the fitted curve is shown).

containing samples yielded values which were similar to what was obtained in the first series of experiments (results not shown). Upon $MgCl_2$ deprivation, the high-affinity binding site was no longer detectable and the shape of the curve was clearly monophasic and indistinguishable from the isoproterenol competition binding curves obtained in the presence of GppNHp (shown for the competition binding curve at 30–37°C only). In the experiment without $MgCl_2$, the effect of changing the temperature was small and comparable to the effect on the low-affinity binding site studied in the first series of experiments: a 1.2 to 1.5 fold increase in K_i value was calculated.

4. Discussion

The study of the thermodynamics of the binding of ligands to adrenoceptors has yielded valuable information on the binding of antagonists and agonists to a large number of adrenoceptor systems (Raffa and Porreca, 1989). The investigation of the β-adrenergic system was probably the first to be described by the group of Molinoff (Weiland et al., 1979). Using competition binding experiments, these authors described the binding of several agonists and antagonists to β-adrenoceptors and the effect of changing the temperature; first in turkey erythrocytes (Weiland et al., 1979), subsequently in different organs of the rat (Weiland et al., 1980) and in cultured L6 myoblasts (Contreras et al., 1986). The main conclusions were that agonist binding was enthalpy-driven, due to both initial binding and agonist-specific isomerization of the adrenoceptor, thereby allowing interaction with the G-protein, while antagonist binding was entropy-driven representing initial binding only. Furthermore, it was concluded that the high-affinity agonist binding site was much more sensitive to temperature changes than the low-affinity binding site. More recently, the interaction of the β_2-selective agonist broxaterol to rat lung membranes was studied and these authors concluded, similarly to Contreras et al. (1986), that the agonist binding is enthalpy-driven, while antagonist binding is entropy-driven (Sala et al., 1991).

We found that in our experimental conditions, the high-affinity binding of isoproterenol is entropy-driven, while the binding of the agonist to the low-affinity binding site and to the binding site detectable in the presence of GppNHp (further defined as GppNHp-sensitive binding site) is enthalpy-driven. The binding of the antagonist is, as found by others, entropy-driven.

Although our results are not compatible with the current opinion on the thermodynamics of agonist binding to β-adrenoceptors, it has to be mentioned that other G-protein coupled adrenoceptors display similarities to what we find (Hitzemann, 1988). Binding of the agonist phenylpropyladenosine to the high-affinity binding of the R_i-adenosine receptor site was found to be entropy-driven while binding to the low-affinity binding site and to the GppNHp-sensitive agonist binding site displayed favorable enthalpy changes (Lohse et al., 1984). The thermodynamic properties of α_2-adrenoceptors were found to be temperature dependent: at low temperatures, agonist and antagonist binding were both entropy-driven while at 30°C, high-affinity binding of agonist was enthalpy-driven (Lohse et al. 1985). Hence, the thermodynamic properties of α_2-adrenoceptors at physiological temperatures are comparable to what we found for β-adrenoceptors.

Some of the discrepancies between our results and those obtained by others could be due to the use of $MgCl_2$ and NaCl. There is apparently a lot of confusion in the literature on the role of these ions in agonist binding. It was demonstrated before that magnesium ions are essential for obtaining high-affinity binding in membranes from HL60 cells (Gierschik et al., 1989), from S 49 lymphoma cells (Birnbaumer, 1990), in permeabilized human lymphocytes (Feldman, 1988) and for agonist induced stimulation of adenylate cyclase (Abrahamson and Molinoff, 1984). However, Contreras et al. (1986) in their experiments with L6 myoblasts, no magnesium is used in the isoproterenol competition binding, yet high-affinity binding was detected. Similarly, in the experiments with different rat tissues (Weiland et al., 1980), magnesium was absent during the ligand binding experiments but present during the stimulation of adenylate cyclase. In the experiments with broxaterol (Sala et al., 1991), no $MgCl_2$ was used. The latter authors included 100 μM GTP in all their preparations, which, evidently, excludes any conclusions on the thermodynamic properties of the high-affinity binding site. Our own experiments (second series) clearly indicated that for rat lung, the presence of Mg^{2+} in the incubation mixture is essential to detect the high-affinity binding site. The disappearance of high-affinity binding site in the absence of $MgCl_2$ is not due to secondary phenomena such as the solubilization of G-proteins, since in our first series of experiments, tissues were prepared without but incubated with Mg^{2+} and yet high-affinity binding is detected. Hence, preparing the crude membrane fraction without $MgCl_2$ is not destructive for the high-affinity adrenoceptor–G-protein interaction.

Classical thermodynamics related to receptor-ligand binding studies interpret positive changes of enthalpy as the result of the formation of bonds either directly with the ligand (agonist or antagonist) or indirectly during conformational changes at other sites in the receptor molecule. In G-protein containing membranes, the binding of agonists to the high-affinity binding site not only causes changes in the conformation of the adrenoceptor but also initiates the interaction between the adrenoceptor and the G-protein, and

between G-protein and adenylate cyclase. The latter is also an energy consuming process which can only be seen under favorable conditions, and since Mg^{2+} are essential for the activation of adenylate cyclase, the interaction between G-proteins and the enzyme can only be observed in the presence of $MgCl_2$ (Abrahamson and Molinoff, 1984). The total energy consumption related to the agonist high-affinity binding is therefore the sum of several parameters: isomerization, G-protein coupling and subsequent activation of adenylate cyclase. Our results suggest that if the experimental conditions are chosen so as to prevent the interaction with adenylate cyclase, high-affinity binding is not longer detectable and is converted to low-affinity binding. This low-affinity binding site has only the capacity to bind the agonist and is not able to transmit the information to the effector system; in this case the agonist-receptor interaction is found to be enthalpy-driven with a small increase in entropy. From a thermodynamic point of view, the binding of an agonist to the low-affinity binding site and the binding of an antagonist to this site are very similar. This could suggest that, if no information is transmitted, the extra energy for coupling to the G-protein is not delivered, resulting in a negative enthalpy and a lower entropy change. It is also evident that in order to get negative free energy, there is a need for a large increase in entropy, which is explained either as a solvent effect, the liberation of water molecules or the unfolding of the interacting proteins (Franklin, 1980; Miklavc et al., 1990). This interpretation is apparently in contrast with the results obtained by Cerione et al. (1984) who suggested that the presence of pure β-adrenoceptors and G_s-proteins, when inserted into phospholipid vesicles and using $MgCl_2$ in the incubation medium, is sufficient to obtain high-affinity agonist binding, although no thermodynamic analysis of the binding was performed. However, it has to be remembered that in this highly artificial model comparisons with the in vivo situation in the plasma membrane remain uncertain. This doubt is further strenghtened by the fact that in their experiments, the ratio between receptor and G-protein was approximately one, while in membranes prepared from tissues or cells in culture, this ratio is much higher (Alousi et al., 1991; Levis and Bourne, 1992).

Another difference in experimental conditions between previous literature data and the results mentioned here is that in the experiments mentioned above (Weiland et al., 1979; Weiland et al., 1980; Contreras et al., 1986), high concentrations of sodium ions were present, in contrast to our preparations. It has been discussed before that for α_2-adrenoceptors (Lohse et al., 1985) and for opiate adrenoceptors (Hitzeman, 1988) sodium ions could play a role in the thermodynamic behavior, and even in relation to the interaction between NaCl and $MgCl_2$, it was suggested that both have opposite effects on the affinity of the binding of agonists to β-receptors. It is however clear from the literature that the effect is primarily quantitative. Indeed, addition or omission of NaCl can cause changes in ΔH or ΔS without, however, changing the reaction from enthalpy-driven to entropy-driven. We therefore believe that the difference in experimental condition cannot explain the discrepancy in the results.

We conclude that the thermodynamic properties of the β-adrenergic high and low-affinity binding sites for isoproterenol differ and that binding of the agonist at the high-affinity binding is characterized by an increase in enthalpy compensated by a large entropy increase. High-affinity agonist binding to β-adrenoceptors requires the interaction between adrenoceptor, G-protein and adenylate cyclase.

5. Acknowledgments

The authors thank Dr. M. Bogaert and Dr. M. Joniau for their critical review of the text. The financial support of the Fund for Medical Scientific Research (Belgium), grant 3.9006.87 is acknowledged. P.V. is a former student of the Belgian I.W.O.N.L. A.B. is the recipient of a grant from the Belgian I.W.O.N.L.

6. References

Abrahamson, S.N. and P.B. Molinoff, 1984, In vitro interactions of agonists and antagonists with β-adrenergic adrenoceptors, Biochem. Pharmacol. 33, 869.

Alousi, A.A., Jasper, J.R., Insel, P.A. and H.J. Motulsky, Stoichiometry of receptor-G_s-adenylate cyclase interactions, FASEB J. 5, 2300.

Cheng, Y.-C. and W.H. Prusoff, 1973, Relation between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) on an enzymatic reaction, Biochem. Pharmacol. 22, 3099.

Contreras, M.L., B.B. Wolfe and P.B. Molinoff, 1986, Thermodynamic properties of agonist interaction with the β-adrenergic adrenoceptor-coupled adenylate cyclase system. I. High and low affinity states of agonist binding to membrane-bound beta adrenergic adrenoceptors, J. Pharmacol. Exp. Ther. 237, 154.

Feldman, R.D., 1988, β-Adrenergic desensitization reduces the sensitivity of adenylate cyclase for magnesium in permeabilized lymphocytes, Mol. Pharmacol. 35, 304.

Cerione, R.A., Codina, J., Benovic J.L., Lefkowitz, R.J., Birnbaumer, L. and M.G. Caron, 1984, The mammalian β_2-adrenergic receptor: reconstitution of functional interactions between pure receptor and pure stimulatory nucleotide binding protein of the adenylae cyclase system, Biochemistry, 23, 4519.

Gierschik, P., Steisslinger, M., Sidiropoulos, D., Herrmann, E. and K.H. Jakobs, 1989, Dual Mg^{2+} control of formyl-peptide-receptor-G-protein interaction in HL60 cells. Evidence that the low-agonist-affinity receptor interacts with and activates the G-proteins, Eur. J. Biochem. 183, 97.

Hitzemann, R., 1988, Thermodynamic aspects of drug-receptor interactions, Trends Pharmacol. Sci. 9, 408.

Fraeyman N. and P. vanscheeuwijck, 1991, Effect of age on the kinetics of the binding of iodocyanopindolol to a membrane preparation of rat lungs, J. Recept. Res. 11, 985.

Franklin, T.J., 1980, Binding energy and the activation of hormone adrenoceptors, Biochem. Pharmacol. 29, 853.

Levis, M.J. and H.R. Bourne, 1992, Activation of the α subunit of G_s in intact cells alters its abundance, rate of degradation and membrane avidity, J. Cell Biol. 119, 1297.

Helmreich, E.J.M. and T. Pfeuffer, 1985, Regulation of signal transduction by β-adrenergic hormone adrenoceptors, Trends Pharmacol. Sci. 6, 438.

Hitzemann, R., 1988, Thermodynamic aspects of drug-adrenoceptor interactions, Trends Pharmacol. Sci. 9, 408.

Levitzki, A., 1988, From epinephrine to cAMP, Science, 241, 800.

Lohse, M.J., V. Lenschow and U. Schwabe, 1984, Two affinity states of R_i adenosine adrenoceptors in brain membranes, Mol. Pharmacol. 26, 1.

Lohse, M.J., K.-N. Klotz and U. Schwabe, 1985, Effects of temperature and membrane phase transition on ligand binding to α_2-adrenoceptors of human platelets, Mol. Pharmacol. 29, 228.

Miklavc, A., D. Kocjan, J. Mavri, J. Koller and D. Hadzi, 1990, On the fundamental difference in the thermodynamics of agonist and antagonist interactions with β-adrenergic receptors and the mechanism of entropy-driven binding, Biochem. Pharmacol. 40, 663.

Minuth, M. and K.H. Jacobs, 1986, Sodium regulation of agonist and antagonist binding to β-adrenoceptors in intact and N_s-dificient membranes, Naunyn-Schmiedeberg's Arch. Pharmacol. 333, 124.

Motulsky, H.J., 1987, GraphPAD; plot, analyze data and digitize graphs. Ed. DiMaggio M., San Diego, ISI-software.

Raffa, R.B. and F. Porreca, 1989, Thermodynamic analysis of the drug-adrenoceptor interaction, Life Sci. 44, 245.

Sala, R., E. Moriggi, D.D. Bella and A. Carenzi, 1991, The specific binding of broxaterol, a new β_2-selective agonist, to β-adrenoceptors, Eur. J. Pharmacol. 203, 17.

Snedecor, G.W. and W.G. Cochran, 1973, Curvilinear regression, Statistical methods, Iowa State University Press, Chapter 15, 447.

Vanscheeuwijck, P. and N. Fraeyman, 1989a, Evaluation of a semi-automatic cell harvester filtration for the determination of β-adrenoceptors in human mononuclear leukocytes, J. Pharmacol. Methods 21, 299.

Vanscheeuwijck, P., E. Van de Velde and N. Fraeyman, 1989b, Effect of aging on properties and function of β-adrenoceptors in rat lung, Eur. J. Pharmacol., Mol. Pharmacol. 172, 373.

Weiland, G.A., K.P. Minneman and P.B. Molinoff, 1979, Fundamental difference between the molecular interactions of agonists and antagonists with the β-adrenergic adrenoceptor, Nature, 281, 114.

Weiland, G.A., K.P. Minneman and P.B. Molinoff, 1980, Thermodynamics of agonist and antagonist interactions with mammalian β-adrenergic adrenoceptors, Mol. Pharmacol. 18, 341.