Non classical, multiple-site interaction of [³H]-prazosin with the α_1 -adrenoceptor of intact BC₃H₁ cells

¹Fritz Sladeczek, *Christopher J. Kirk, Joël Bockaert & ²Bernard H. Schmidt

Centre CNRS-INSERM de Pharmacologie-Endocrinologie, Rue de la Cardonille, F-34094 Montpellier Cedex 2, France and *Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT

1 In intact BC₃H₁ cells the EC₅₀ of noradrenaline (NA) for the inositol phosphate response measured at 37°C (EC₅₀ = 193 nM) was much lower than its apparent dissociation constant ($K_i^{37^\circ\text{C}}$ = 83.211 μM) determined at this temperature by [³H]-prazosin binding.

2 After pretreatment of the cells with NA at 37°C for 45 min, the time used in binding assays at this temperature, this difference between EC₅₀ and $K_i^{37^\circ\text{C}}$ did not decrease significantly. An agonist-induced reduction in α_1 -adrenoceptor affinity can therefore not explain the very high $K_i^{37^\circ\text{C}}$ value.

3 NA pretreatment at 37°C decreased the number of [³H]-prazosin binding sites (assessed by whole cell binding at 2°C) by only 49%; not by 100%, the value expected if agonist-induced receptor internalization were the origin of the very low $K_i^{37^\circ\text{C}}$.

4 The EC₅₀ of NA for the inositol phosphate response in the presence of 156 pM [³H]-prazosin was 1.841 μM but the IC₅₀ of NA for the inhibition of [³H]-prazosin binding (126 pM) was 316 μM . As there is no α_1 -adrenoceptor reserve in these cells we propose that at 37°C [³H]-prazosin interacts, not only with the catecholamine recognition site (site 1) of the receptor, but also reacts weakly with another site from which it cannot be directly displaced by catecholamine-like substances (site 2).

Introduction

The apparent affinities of noradrenaline (NA) for the [³H]-prazosin binding sites on intact BC₃H₁ cells are much higher in the cold than at 37°C (Sladeczek *et al.*, 1983). In a recent study we found that the EC₅₀ values at 37°C for several adrenoceptor agonists which stimulate inositol phosphate accumulation were situated between their dissociation constants at 2°C ($K_i^{2^\circ\text{C}}$) and at 37°C ($K_i^{37^\circ\text{C}}$) (Sladeczek *et al.*, 1988b). The $K_i^{37^\circ\text{C}}$ values were all in the same range (0.1 to 1 mM) and showed no simple correlation with the EC₅₀ values. The $K_i^{2^\circ\text{C}}$ values for almost all of these agonists, correlated linearly with the EC₅₀ values. They were about 20–100 times lower than the

respective EC₅₀ values. This led us to suggest that the $K_i^{2^\circ\text{C}}$ values might correspond to a high affinity state of the drug recognition site for catecholamines. As this high affinity site was not found in a study in which another radioligand ([¹²⁵I]-BE 2254) was used (Hoyer, 1984), we proposed a model in which this state was induced by [³H]-prazosin.

However, the difference between EC₅₀ and $K_i^{37^\circ\text{C}}$ values still needs to be explained. Agonist degradation is not at the origin of these high $K_i^{37^\circ\text{C}}$ values. Several arguments exclude the possibility of lysosomal uptake of [³H]-prazosin being the origin of the low apparent agonist affinities observed in whole cell competition experiments. Inhibitors of this uptake did not affect [³H]-prazosin binding in intact BC₃H₁-cells (Sladeczek *et al.*, 1983). Dissociation of specific [³H]-prazosin binding from intact BC₃H₁-cells is monophasic (Amitai *et al.*, 1984) and

¹ Author for correspondence.

² Present address: Troponwerke GmbH & Co KG, Berliner Strasse, 156, D-5000, Köln 80, Postfach 801060, F.R.G.

not multiphasic as would be expected for a combined binding/uptake phenomenon. The number of [^3H]-prazosin binding sites in intact BC_3H_1 -cells is very close to that found in broken cell preparations (Sladeczek *et al.*, 1983).

Another explanation for a much lower EC_{50} than $K_i^{37^\circ\text{C}}$ value is an important receptor reserve. This would mean in our case that the occupation of less than 1% of the α_1 -adrenoceptors of intact BC_3H_1 -cells by NA is sufficient to elicit the full response to inositol phosphate. However, in a previous paper we demonstrated that there is an almost linear coupling between receptor occupation and IP-response measured in our conditions (Sladeczek *et al.*, 1988b). The 'true' affinity of agonists at 37°C corresponds therefore to their EC_{50} and not to their $K_i^{37^\circ\text{C}}$ values.

What could be the origin of the very high $K_i^{37^\circ\text{C}}$ values observed? Figure 1 depicts three possible mechanisms which might account for these unusual $K_i^{37^\circ\text{C}}$ values.

(a) Agonist-induced reduction in affinity. The agonist initiates the IP-response with an affinity corresponding to its EC_{50} value. During the 45 min incubation period the affinity of the receptor for the agonist would shift to its $K_i^{37^\circ\text{C}}$ value. As the $K_i^{37^\circ\text{C}}$ values for all the agonists tested were very similar (Sladeczek *et al.*, 1988b) this means that the drug recognition site of the α_1 -adrenoceptor would lose its drug selectivity after prolonged incubation with an agonist.

(b) The second possible explanation for the differences in the EC_{50} and $K_i^{37^\circ\text{C}}$ values is that agonists might induce internalization of the receptor. Lipophilic substances like prazosin are thought to be able to cross the structures separating the internalized receptor from the extracellular medium whereas hydrophilic substances like NA, cannot. In competition experiments at 37°C , internalized receptors would be much less accessible to NA than to [^3H]-prazosin. This could explain the very high concentrations of NA required to inhibit [^3H]-prazosin binding at this temperature. In the cold, internalization cannot occur and real competition between [^3H]-prazosin and NA for plasma membrane receptors is observed. In this model $K_i^{37^\circ\text{C}}$ values would depend on the agonist-activity (necessary for internalization) and the lipophilicity (necessary for penetration of the lipid bilayer) of the competing drug and on the lipophilic character of the radioligand used.

Agonist-induced receptor internalization is a well-known phenomenon for a great number of receptors including: insulin, EGF (epidermal growth factor) (Goldstein *et al.*, 1979; Steinman *et al.*, 1983) or β -adrenoceptors (Hertel & Perkins, 1984; Sibley & Lefkowitz, 1985). Agonist-induced receptor loss has

a Agonist-induced reduction in affinity

b Agonist-induced receptor internalisation

c Complex agonist ↔ ligand interaction

Figure 1 Three simple models which could explain the temperature dependence of apparent receptor affinities for noradrenaline (NA) in BC_3H_1 cells. The question mark between low affinity receptor and 'response' in (a), indicates that the desensitized receptor may still be coupled, or already uncoupled from the effector system. For explanation of the diagrams see Introduction and Results sections. Abbreviations: R = receptor, A = agonist, *P = [^3H]-prazosin.

also been described for α_1 -adrenoceptors in BC_3H_1 -cells (Hughes & Insel, 1986). It was a very slow process, only detectable after 1 h. However, in these experiments the receptor number was assessed by [^3H]-prazosin saturation experiments on membrane fractions. In addition to cell surface receptors, internalized but not yet degraded receptors would be detected by this method. More recently a much more rapid ($t_{1/2} = 2$ min) NA-induced disappearance of [^3H]-prazosin binding sites was detected in DDT_1 cells in the cold (Leeb-Lundberg *et al.*, 1987; Fratelli & De Blasi, 1987). It was suggested that this rapid disappearance of [^3H]-prazosin binding sites was due to receptor sequestration, which is often

Table 1 Effect of temperature and pretreatment with noradrenaline (NA) and phorbol 12-myristate 13-acetate (PMA) on [³H]-prazosin binding characteristics

	K_D (pM)	B_{max} (sites per cell)	Number of experiments
Control (37°C)	26.4 ± 3.6	18740 ± 2782	5
Control (2°C)	44.7 ± 8.5	18379 ± 2674	8
Control (2°C, washed)	46.4 ± 14.1	16802 ± 3372	3
NA-pretreatment	61.7 ± 11.9	8587 ± 2373	3
Control (2°C, DMSO)	31.0 ± 11.7	15657 ± 2408	3
PMA-pretreatment	75.3 ± 13.7	16380 ± 2650	3

K_D and B_{max} values were determined in saturation experiments as described in Methods. For PMA pretreatment, cells were incubated at 37°C for 10 min with 100 nM PMA. Dishes were then put on ice and saturation experiments initiated 30 min later. For NA desensitization experiments, cells were incubated for 45 min at 37°C with 10 μ M NA and then put on ice. Thirty min later, dishes were washed three times with 1 ml ice-cold washing buffer and saturation experiments were initiated thereafter. Incubation time at 2°C was 7 h. Control (2°C, washed) dishes were treated as NA dishes. 0.1% DMSO was added instead of PMA to control (2°C, DMSO) dishes and they were then treated as PMA dishes.

observed after stimulation of adenylate cyclase-coupled receptors (Hertel & Perkins, 1984; Sibley & Lefkowitz, 1985). If almost 100% of the α_1 -adrenoceptors in BC₃H₁ cells disappeared from the cell surface during the 45 min incubation period used for the binding assays at 37°C, the very high $K_i^{37^\circ\text{C}}$ values could be explained by this phenomenon.

(c) A third explanation for the differences in the EC_{50} and $K_i^{37^\circ\text{C}}$ values could be that prazosin interacts at 37°C (but not at 2°C), with both the catecholamine recognition site and with a second site, not accessible to catecholamines. These agonists would therefore compete at 37°C with [³H]-prazosin in a non-classical manner and with artificially low affinity. Antagonists such as phentolamine had access to both sites. This would explain why there was no great difference between the $K_i^{37^\circ\text{C}}$ and the $K_i^{2^\circ\text{C}}$ values for these substances (Sladeczek *et al.*, 1983). In this model the $K_i^{37^\circ\text{C}}$ values of the competing drugs would depend on their structure and their ability to interact with both or with only one of the [³H]-prazosin binding sites. The purpose of the present study was to investigate which of these hypotheses could explain the differences between the EC_{50} and $K_i^{37^\circ\text{C}}$ values of agonists for α_1 -adrenoceptors of intact BC₃H₁ cells.

Methods

The BC₃H₁ muscle cell line was a gift from Dr J. Patrick (The Salk Institute, San Diego, CA, U.S.A.). The cells were grown in 35 mm tissue culture dishes

as previously described (Mauger *et al.*, 1982) with the exception that no penicillin or streptomycin sulphate was used. Cells were used 7 to 10 days after seeding without any change of culture medium.

[³H]-prazosin binding experiments on intact BC₃H₁ cells were performed as described previously (Sladeczek *et al.*, 1983). Briefly: culture medium was replaced by 2 ml of HEPES-buffered saline and cells were allowed to equilibrate for at least 15 min at the respective incubation temperature. The assay was initiated by addition of the radioligand and the indicated drugs. The incubation periods were 7 h at 2°C, 5 h at 6°C, 4 h at 10°C, 2 h 30 min at 14°C, 60 min at 18°C and 45 min at 22, 26, 30 and 37°C; it had been checked previously that these periods were sufficient for equilibrium to be achieved. The incubation was terminated by aspiration of the incubation medium followed by washing the culture dishes three times with 3 ml of phosphate buffered saline. Cells were collected from the dishes by the addition of 1 ml of 0.5% Triton X-100/0.2 M NaOH, transferred into a counting vial, neutralised and counted in scintillation fluid. Specific binding was defined as the binding displaced by 10 μ M phentolamine. The maximal number of binding sites was almost the same at 2 and 37°C (Table 1).

For IP experiments, cells were seeded in 35 mm plastic dishes in 1.5 ml culture medium containing 1.33 μ Ci ml⁻¹ of [³H]-inositol. Before each experiment the culture medium was replaced by HEPES-buffered saline and the cells were pre-incubated for 10 min with 5 mM Li₂SO₄ in order to block IP-degradation (Sherman *et al.*, 1981; Berridge *et al.*, 1982). The HEPES buffer did not contain any inositol. Reactions were started by the addition of ligands, yielding the indicated concentrations in a

final volume of 1 ml. In standard experiments the reaction was stopped after 45 min by replacing the incubation medium with 5% perchloric acid (PCA). The IPs were extracted as described by Bone *et al.* (1984) and separated by Dowex anion exchange chromatography (Sladeczek *et al.*, 1988a,b).

Results

In order to test the three hypothesis which might explain the low apparent agonist affinities at 37°C (Figure 1), we performed the following experiments.

Agonist induced reduction in affinity

If an agonist-induced reduction in affinity (Figure 1a) was at the origin of the difference between the EC_{50} and $K_i^{37^\circ C}$ values, either a cellular response mediated by NA should not be detectable any more (receptor uncoupled from its effector system) after pretreatment of the cell with NA at 37°C or, if the response could still be stimulated by NA the EC_{50} value should approach the $K_i^{37^\circ C}$ value.

In Figure 2 we compare the effects of NA on [3H]-prazosin binding at 2°C and 37°C with its ability to induce the formation of IPs at 37°C. Pretreatment of the cells with the indicated concentrations of NA resulted in a very weak rightward shift of the dose-response curve for IP accumulation, but the concentrations of NA required to stimulate IP formation remained about 1000 fold less than those required to compete with [3H]-prazosin binding. Furthermore, the maximal IP-response to NA was not significantly changed by the pretreatment, indicating that NA did not induce desensitization of this response.

A more direct way to test the desensitization hypothesis is by direct binding experiments. It has been shown that, in intact hepatocytes, adrenaline and NA exhibit biphasic competition curves when [3H]-prazosin is used as a ligand at 37°C (Schwarz *et al.*, 1985; Corvera *et al.*, 1986). A small fraction of the [3H]-prazosin sites showed high affinity (9–25 nM) for the agonists whereas the majority of the sites exhibited low agonist affinities (about 2000 nM). Pretreatment of the cells with 100 nM of the phorbol ester phorbol 12-myristate 13-acetate (PMA) shifted all the sites to the low affinity state (Corvera *et al.*, 1986). In the cold only high affinity sites were detected (Schwarz *et al.*, 1985). It was concluded that the agonist affinity measured in the cold corresponds to the initial affinity of the receptor for agonists (Schwarz *et al.*, 1985) and activation of protein

Figure 2 Comparison of the effects of noradrenaline (NA) on [3H]-prazosin competition experiments at 2°C ($\square$) and 37°C ($\blacktriangle$) and inositol phosphate (IP) accumulation under normal ($\circ$) and desensitized ($\bullet$) conditions. IP accumulation was measured after preincubating BC $_3$ H $_1$ cells for 45 min in the presence ('desensitized') ($\bullet$) or absence (control) ($\circ$) of the indicated concentrations of NA at 37°C. IP accumulation was initiated by addition of 10 mM LiSO $_4$ ('control') or 10 mM LiSO $_4$ and NA at the indicated concentrations ('desensitized'). Reactions were stopped after a further 45 min incubation by replacing the medium with 5% perchloric acid. IP extraction and separation and [3H]-prazosin binding experiments were performed as described in Methods. Results are expressed as a percentage of the maximal effect of NA and are the means of at least 6 independent determinations performed on at least 3 different culture preparations. Vertical lines show s.e.mean. The mean concentration of [3H]-prazosin used in these experiments was 126 ± 10 pM.

kinase C by PMA at 37°C provoked receptor phosphorylation, thus transforming all high affinity sites into low affinity sites (Corvera *et al.*, 1986).

If the high affinity sites detected at 2°C in BC $_3$ H $_1$ cells corresponded to the PMA-sensitive sites in hepatocytes, pretreatment of the cells at 37°C with 100 nM PMA should transform all the high affinity sites into low affinity agonist sites. For this reason, we pretreated BC $_3$ H $_1$ cells at 37°C for 10 min with 100 nM PMA, a concentration needed to obtain maximal phosphorylation of α_1 -adrenoceptors in DDT $_1$ cells (Leeb-Lundberg *et al.*, 1985; 1987). As in DDT $_1$ cells, this protocol inhibited 100% of the α_1 -adrenoceptor-mediated response in our system (data not shown). In NA competition experiments at 2°C, we obtained a K_i of 9.5 nM, corresponding to the usual high affinity state at this temperature. This was not due to a reversal of the PMA-induced desensitization, since the PMA blockade of the IP

response was still effective after the 7 h incubation period at 2°C (data not shown). Furthermore, cells preincubated at 37°C for 45 min with varying concentrations of NA (from 100 pM to 1 mM) and then put on ice, showed the classical high affinity NA site (11.3 nM) in competition experiments with [³H]-prazosin.

Model 1a does not therefore explain the differences observed between EC₅₀ and K_i^{37°C} values.

Agonist-induced receptor internalisation

In order to test the internalisation hypothesis (Figure 1b) we performed [³H]-prazosin saturation experiments on whole cells in the cold after pretreating them for 45 min at 37°C with a maximally effective concentration of NA (10 μ M). The results shown in Table 1 indicate a loss of about 49% of the receptors with no significant change in their K_D for [³H]-prazosin. The remaining surface receptors kept their high affinity for NA in the cold (K_i^{2°C} = 4 nM). If such a receptor sequestration was the reason for the appearance of the low affinity NA state at 37°C during the incubation period of 45 min, in equilibrium binding experiments at this temperature we should obtain a clearly biphasic competition curve. The results presented in Figure 2 show that this is not the case. Model 1b is therefore not able to explain the high temperature dependence of the apparent affinities of NA for α_1 -adrenoceptors in intact BC₃H₁ cells.

The concentration of PMA (100 nM) which was maximally active in phosphorylating the α_1 -adrenoceptor in DDT₁ cells (Leeb-Lundberg *et al.*, 1985) decreased the affinity of the receptor for [³H]-prazosin by a factor of 2, but had no effect on the number of maximal binding sites (Table 1) in our system.

Complex agonist-ligand interactions

Another possibility which could explain the temperature-dependence of the affinities for NA is shown in Figure 1c. Prazosin and NA have quite different structures. Their structural similarities are limited to the catechol head region in which the hydroxyl groups of NA are exchanged by methoxy groups in the prazosin molecule. The hydroxylated amino ethyl tail of NA is replaced in prazosin by a rigid pyrimidine cycle containing, in position 2, a large tail consisting of a cyclic aliphatic diamine, a ketone and a furane group. It is therefore possible that the prazosin molecule may interact with both, the catecholamine acceptor site (site 1 in Figure 1c) and with another site accepting its large tail region

(site 2 in Figure 1c). NA might only be able to displace the part of the prazosin molecule fixed in site 1 and the radioligand would remain attached to the receptor by its interaction with site 2. There would be, as in every equilibrium reaction, continuous association and dissociation of the drugs interacting with the receptor. Thus as soon as NA dissociated from site 1, prazosin would very quickly reassociate with this site due to the fact that it remained attached to site 2. In this case, NA would displace prazosin from its receptor (in a way detectable in binding experiments) only when its concentration was high enough to produce a mean NA occupation time of site 1 greater than the prazosin dissociation rate from site 2. If site 2 was not accessible at 2°C much lower concentrations of NA would be able to displace prazosin from its receptor.

A way to test this hypothesis is to perform NA dose-response curves in the presence and absence of prazosin at 37°C. Figure 3 shows that 156 pM [³H]-prazosin shifted the IP dose-response curves in an almost competitive manner to the right. The observed EC₅₀ of 1585 nM was very close to the EC₅₀ of 1841 nM, calculated by the Cheng Prusoff equation (Cheng & Prusoff, 1973). Therefore at 37°C NA is able to compete in an almost classical manner with [³H]-prazosin for the biologically active site

Figure 3 Effect of [³H]-prazosin on the dose-response curves for noradrenaline (NA)-induced formation of inositol phosphate (IP) in BC₃H₁ cells. Cells were preincubated for 45 min in the absence (control, ■) or presence (●) of 156 pM [³H]-prazosin and experiments were performed as described in Methods. Results are expressed as % of maximal effect of NA and are the means of six independent determinations performed on three different culture preparations. Vertical lines show s.e.mean. EC₅₀ values = 263 nM, control, and 1585 nM in the presence of prazosin.

Figure 4 $[^3\text{H}]$ -prazosin/noradrenaline competition experiments at different temperatures in BC_3H_1 cells: (○) 2°C , (+) 6°C , (△) 10°C , (□) 14°C , (☆) 18°C , (●) 22°C , (▲) 26°C , (◇) 30°C and (■) 37°C . Results are expressed as % of specifically bound $[^3\text{H}]$ -prazosin and are the means of eight independent determinations performed on four different culture preparations.

(site 1) (Figure 3), but is much less efficient in displacing 126 pM $[^3\text{H}]$ -prazosin from the receptor at this temperature (Figure 2, $\text{IC}_{50} = 316\text{ }\mu\text{M}$).

In order to determine if the appearance of site 2 is an all or nothing phenomenon which occurs at a particular temperature or appears progressively as the temperature increases, we performed NA competition experiments at different temperatures. The competition curves obtained at 2°C and 6°C were almost identical (Figure 4). Between 10°C and 30°C , there was a progressive shift to lower apparent affinities of NA. At 30°C , the apparent affinity of NA was the same as that usually observed at 37°C .

Discussion

BC_3H_1 cells exhibit a loss of 49% of $[^3\text{H}]$ -prazosin binding sites, compared to those measured in the cold, after pretreatment with NA for 45 min at 37°C . This loss of binding sites was not reproduced by application of PMA (Table 1) at a concentration (100 nM) which completely inhibited the IP-response in BC_3H_1 cells. A similar observation was recently made in DDT_1 -cells (Leeb-Lundberg *et al.*, 1987), where the kinetics of PMA-induced α_1 -adrenoceptor phosphorylation paralleled the kinetics of loss of the inositol phospholipid response. It can therefore be concluded that the failure of protein kinase C to induce α_1 -adrenoceptor sequestration is not limited to DDT_1 cells but seems to be a general phenomenon.

There is one difference between our work on BC_3H_1 cells and the work on DDT_1 -cells. Leeb-Lundberg *et al.* (1987) observed a significant reduction of the agonist-induced IP response in DDT_1 -cells after pretreatment with NA. In BC_3H_1 cells we saw no such effect after a similar pretreatment (Figure 2). However, it should be noted that Leeb-Lundberg *et al.* (1987) utilized shorter periods of pretreatment and stimulation and measured ^{32}P labelling of the phospholipids rather than the accumulation of IPs, which is a more direct measure of lipid hydrolysis.

Non-equilibrium competition experiments have previously been done to demonstrate an initial high affinity agonist state of α_1 -adrenoceptors (Toews, 1987; Fratelli *et al.*, 1987). However the postulates at the origin of such a procedure have never been seriously investigated. One of these postulates is that the radioligand and agonist both interact in a simple monostep procedure ($\text{Drug} + \text{Receptor} = \text{Drug-Receptor}$) with the receptor. However, our results indicate that the $[^3\text{H}]$ -prazosin α_1 -adrenoceptor interaction is much more complicated. The K_D of an equilibrium reaction is defined as $K_D = k_-/k_+$. The temperature dependency of the rate constant k_+ is given by the Arrhenius law: $k_+ = A_+ e^{-E_{a+}/RT}$ with: A_+ = activation energies of association or dissociation, T = absolute temperature ($^\circ\text{K}$) and R = universal gas constant = $1.99\text{ cal mol}^{-1}\text{ K}^{-1}$. The energy liberated or consumed by an equilibrium reaction (ΔG) is given by the equation $\Delta G = RT \ln K_D$. The K_D values listed in Table 1 allow the determination of ΔG liberated by the $[^3\text{H}]$ -prazosin binding reactions at 2°C (-13 kcal mol^{-1}) and at

37°C (−15 kcal mol^{−1}). For a classical one step equilibrium reaction $E_{a-} = E_{a+} + \Delta G$. ΔG was very important in the equilibrium [³H]-prazosin/ α_1 -adrenoceptors. The consequence should be that $E_{a-} \gg E_{a+}$. With increasing temperatures k_- should therefore increase much quicker than k_+ and $K_D^{37^\circ\text{C}} > K_D^{2^\circ\text{C}}$. But in our experiments the $K_D^{37^\circ\text{C}}$ of 26.4 pM was even slightly lower than the $K_D^{2^\circ\text{C}}$ of 44.7 pM. A detailed kinetic study of the [³H]-prazosin α_1 -adrenoceptor interaction indicates that [³H]-prazosin association could be a multistep procedure involving a preassociation state requiring a very high activation energy (Sladeczek *et al.*, unpublished observations). This could explain why K_D of [³H]-prazosin exhibits no important temperature-dependent effect. Non-equilibrium competition experiments are impossible to interpret in such a complicated kinetic system without detailed investigation of association and dissociation kinetics of all the drugs used. We therefore used exclusively equilibrium binding studies in the present work.

The results presented here show that neither agonist-induced reduction in affinity nor agonist-induced receptor internalization could account for the very low apparent agonist affinities observed in whole cell competition experiments performed with [³H]-prazosin. The temperature-dependent effect of the apparent affinities of NA shown in Figure 4 further supports this. It is indeed possible that a conformational change of the receptor structure at the origin of agonist-induced reduction in affinity and receptor internalization are impossible at low temperatures, due to the fact that the membrane is in its solid, frozen state. But as soon as the threshold temperature between the solid and liquid state of the membrane is reached, we should observe an immediate appearance of the low affinity state. The appearance of the low affinity state with increasing temperatures should therefore be an all or nothing phenomenon. The progressive appearance of this low affinity state shown in Figure 4 is best explained by the multisite interaction model depicted in Figure 1c. In this case [³H]-prazosin would interact at 2°C and 6°C exclusively with site 1. At higher temperatures the interaction with site 2 progressively appeared. The site 2 region could be part of the receptor molecule. Another possibility is that the site 2 interaction reflects a dissolution of the very lipophilic prazosin molecule in some lipophilic phase of the receptor complex. The progressive nature of the appearance of site 2 with increasing temperature supports such a dissolution phenomenon. If the active site of α_1 -adrenoceptors is, similar to that of β -adrenoceptors, on the lipophilic part of the receptor molecule (Bar-Sinai *et al.*, 1986; Yarden *et al.*, 1986; Dixon *et al.*, 1987a,b; Dohlmann *et al.*, 1988; Kobilka *et al.*, 1988), such a dissolution phenomenon

would not be very surprising. In order to test this hypothesis hydrophilic antagonists for the α_1 -adrenoceptor have to be developed.

However, in intact DDT₁ cells (Toews, 1987) only 52% of the [³H]-prazosin binding sites had a low affinity for NA, whereas 48% had a $K_i^{37^\circ\text{C}}$ of 1.32 μM close to the EC₅₀ value of 0.38 μM for the IP response described by us (Sladeczek *et al.*, 1988b). In [³H]-prazosin competition experiments performed in intact brain slices even low affinity sites for agonists had not been detected (Kendall *et al.*, 1985). When [¹²⁵I]-BE 2254 or [³H]-dihydroergocryptine was used, lower $K_i^{37^\circ\text{C}}$ values for NA were observed than the one described by us in [³H]-prazosin whole cell binding assays (Cornett & Norris, 1982; Cornett *et al.*, 1982; Hoyer, 1984; Leeb-Lundberg *et al.*, 1985). The presence of low affinity interactions is therefore highly dependent on the cell system and the radioligand used.

The interaction of [³H]-prazosin with the active site of α_1 -adrenoceptors in intact BC₃H₁ cells at 37°C is as complex as that depicted in Figure 5, if the [³H]-prazosin-induced high affinity agonist state, we recently postulated in order to explain the unusually high apparent agonist affinities observed at 2°C (Sladeczek *et al.*, 1988b), is still present at 37°C. In this model the bulky radioligand is supposed to be a bifunctional molecule and we suggest that it interacts at higher temperatures with 2 sites of the receptor (site 1 and 2 in Figure 5) with the dissociation constant $K_{D1+2} = K_D^{37^\circ\text{C}} = 26.2$ pM. The interaction of [³H]-prazosin with the drug-specific site 1 (dotted line in state II) could lead to the proposed slight distortion of the active site region (Sladeczek *et al.*, 1988b). This distortion would increase the apparent affinity of the active site for catecholamine like drugs (state II to state III). The high affinity interaction of NA with this site 1 in the presence of [³H]-prazosin would lead to the dissociation of [³H]-prazosin from site 1 (state III to state IV). As a result of the site 2 interaction, the radioligand would remain attached to the receptor (state IV). The high affinity agonist state would therefore not be detected by binding experiments at 37°C. As soon as [³H]-prazosin dissociates from site 1, the receptor would return to its normal agonist state (state V). The fact that NA competes in a classical manner with the receptor in IP experiments (Figure 3) shows that NA is able to stimulate the IP-response via the normal agonist site 1 in state V even in the presence of [³H]-prazosin on site 2. But as the mean occupation time of site 1 by NA is not high enough to allow dissociation of [³H]-prazosin from site 2 before NA dissociates (state V to state VI), low concentrations of the catecholamine are not able to displace [³H]-prazosin in a way detectable in binding experiments (Figure 2). [³H]-prazosin

Figure 5 Schematic representation of the hypothetical interaction of [^3H]-prazosin with the α_1 -adrenoceptor of intact BC_3H_1 cells at 37°C . A detailed description of the model is given in the Discussion. K_i values are the dissociation constants of noradrenaline (NA). EC_{50} is the concentration of NA that evokes 50% of the inositol phosphate (IP) response at 37°C . As there is a linear coupling between receptor occupancy and IP-response (2) the EC_{50} represents the 'true' affinity of NA for its receptor. $K_D^{37^\circ\text{C}}$ is the dissociation constant of [^3H]-prazosin measured at 37°C . K_{D1} , K_{D2} and K_{D1+2} are the dissociation constants characteristic for the interaction of [^3H]-prazosin with site 1, site 2 or site 1 and 2 simultaneously.

remains close to site 1 and reassociates immediately with site 1 as soon as NA dissociates (state VI to state II). Only at very high concentrations can NA occupy site 1 longer than [^3H]-prazosin needs to dissociate from site 2 of the receptor (state V to state VII). Agonists are only able to activate the receptor in its normal configuration (states V and VII). [^3H]-prazosin displaces the NA dose-response curve for IP accumulation to the right (Figure 3) and not to the left, as would be expected if NA could stimulate the IP response via the postulated [^3H]-prazosin-induced high affinity agonist states (states III and IV, which are both transition states).

The [^3H]-prazosin-induced distortion of the active site region of α_1 -adrenoceptors observed at 2°C , and as site 2 interaction at 37°C , seem to depend on the integrity of the α_1 -adrenoceptor transmembrane signal transduction system. Both effects were much lower or even non-existent in systems in which no obvious α_1 -adrenoceptor-mediated IP-response could be detected (Sladeczek *et al.*, 1988b).

For β -adrenoceptors, unusually low agonist affinities on intact cells were also obtained when lipophilic radioligands were used (Porzig *et al.*, 1982;

Staehelin *et al.*, 1983). If binding was done with the hydrophilic ligand CGP-12177 more physiological agonist affinities were found. Non-competitive interactions between agonist and lipophilic ligands have been suggested in order to explain this finding. Interactions of the type shown in Figure 1c for lipophilic ligands with the β -adrenoceptor might also be at the origin of this non-competitive behaviour.

In conclusion, it is predicted that similar situations to the one described in this paper, might exist for other receptors for which very large, lipophilic ligands are used for the assessment of active sites specific for much smaller, hydrophilic natural hormones and neurotransmitters. This could be the case for 5-hydroxytryptamine receptors when using ketanserin as a radioligand, or cholinergic receptors when using quinuclidinyl benzilate as a radioligand. If site 2 type interactions of large artificial ligands is a general feature of high affinity antagonists, this discovery could have important implications in the design of new drugs. Bulky, lipophilic ligands, exhibiting the complex binding behaviour described here and in our previous paper (Sladeczek *et al.*, 1988b), might become precious tools for the defini-

tion of the tertiary structure of the active site regions of functional transmembrane receptors.

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References

- AMITAI, G., BROWN, R.D. & TAYLOR, P. (1984). The relationship between α_1 -adrenergic receptor occupation and the mobilization of intracellular calcium. *J. Biol. Chem.*, **259**, 12519–12527.
- BAR-SINAI, A., ALDOUBY, Y., CHOREV, M. & LEVITSKY, A. (1986). Association of turkey erythrocyte β -adrenoceptors with a specific lipid component. *E.M.B.O. J.*, **5**, 1175–1180.
- BERRIDGE, M.J., DOWNES, C.P. & HANLEY, M.R. (1982). Lithium amplifies agonist dependant phosphatidylinositol responses in brain and salivary glands. *Biochem. J.*, **206**, 587–595.
- BONE, E.A., FRETTE, P., PALMER, S., KIRK, C.J. & MICHELL, R.H. (1984). Rapid accumulation of inositol phosphates in isolated rat superior cervical sympathetic ganglia, exposed to V_1 -vasopressin and muscarinic cholinergic stimuli. *Biochem. J.*, **221**, 803–811.
- CHENG, Y.-C. & PRUSOFF, W.H. (1973). Relationship between the inhibitor constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099–3108.
- CORNETT, L.E. & NORRIS, J.S. (1982). Characterization of the α_1 -adrenergic receptor subtype in a smooth muscle cell line. *J. Biol. Chem.*, **257**, 694–697.
- CORNETT, L.E., BALL, D.W. & NORRIS, J.S. (1982). α_1 -adrenergic receptors of a smooth muscle cell line: Guanine nucleotides do not regulate agonist affinities. *J. Rec. Res.*, **2**, 601–615.
- CORVERA, S., SCHWARZ, K.R., GRAHAM, R.M. & GARCIA-SAINZ, J.A. (1986). Phorbol esters inhibit α_1 -adrenergic effects and decrease the affinity of liver cell α_1 -adrenergic receptors for (–)-epinephrine. *J. Biol. Chem.*, **261**, 520–526.
- DIXON, R.A., SIGAL, I.S., RANDS, E., REGISTER, R.B., CANDELORE, M.R., BLAKE, A.D. & STRADER, C.D. (1987a). Ligand binding to the β -adrenergic receptor involved its rhodopsin-like core. *Nature*, **326**, 73–74.
- DIXON, R.F.A., SIGAL, I.S., CANDELORE, M.R., REGISTER, R.B., SCATTERGOOD, W., RANDS, E. & STRADER, C.D. (1987b). Structural features required for ligand binding to the β -adrenergic receptor. *E.M.B.O. J.*, **6**, 3269–3275.
- DOHLMAN, H.G., CARON, M.G., STRADER, C.D., AMLAIKY, N. & LEFKOWITZ, R.J. (1988). Identification and sequence of a binding site peptide of the β_2 -adrenergic receptor. *Biochemistry*, **27**, 1813–1817.
- FRATELLI, M. & DE BLASI, A. (1987). Agonist-induced α_1 -adrenergic receptor changes. Evidence for receptor sequestration. *FEBS Lett.*, **212**, 149–153.
- FRATELLI, M., MARASCO, O. & DE BLASI, A. (1987). Two subpopulations of α_1 -adrenergic receptors with high and low affinity for agonists: short term exposure to agonists reduced the high-affinity sites. *Biochim. Biophys. Acta*, **930**, 87–96.
- GOLDSTEIN, J.L., ANDERSON, R.G.W. & BROWN, M.S. (1979). Coated pits, coated vesicles and receptor mediated endocytosis. *Nature*, **279**, 679–685.
- HERTEL, C. & PERKINS, J.P. (1984). Receptor-specific mechanisms of desensitization of β -adrenergic receptor function. *Mol. Cell. Endocrinol.*, **37**, 245–256.
- HOYER, D. (1984). Characterization of I-BE 2254 binding to α_1 -adrenergic receptors on intact DDT₁ smooth muscle cells: comparison with membrane binding and correlation with phosphoinositides breakdown. *J. Rec. Res.*, **4**, 51–67.
- HUGHES, R.J. & INSEL, P.A. (1986). Agonist-mediated regulation of α_1 - and β_1 -adrenergic receptor metabolism in a muscle cell line, BC₃H₁. *Mol. Pharmacol.*, **29**, 521–530.
- KENDALL, K.A., BROWN, E. & NAHORSKI, S.R. (1985). α_1 -adrenoceptor-mediated inositol phospholipid hydrolysis in rat cerebral cortex: relationship between receptor occupancy and effects of denervation. *Eur. J. Pharmacol.*, **114**, 41–52.
- KOBILKA, B.K., KOBILKA, T.S., DANIEL, K., REGAN, J.W., CARON, M.G. & LEFKOWITZ, R.J. (1988). Chimeric α_2 -, β_2 -adrenergic receptors: Delineation of domains involved in effector coupling and ligand binding specificity. *Science*, **240**, 1310–1316.
- LEEB-LUNDBERG, L.M.F., COTECCHIA, S., LOMASNEY, J.W., DE BARNADIS, J.F., LEFKOWITZ, R.J. & CARON, M.G. (1985). Phorbol esters promote α_1 -adrenergic receptor phosphorylation and receptor uncoupling from inositol phospholipid metabolism. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 5651–5655.
- LEEB-LUNDBERG, L.M.F., COTECCHIA, S., DE BLASI, A., CARON, M.G. & LEFKOWITZ, R.J. (1987). Regulation of adrenergic receptor function by phosphorylation. I. Agonist-promoted desensitization and phosphorylation of α_1 -adrenergic receptors coupled to inositol phospholipid metabolism in DDT₁ MF-2 smooth muscle cells. *J. Biol. Chem.*, **262**, 3098–3105.
- MAUGER, J.-P., SLADCEK, F. & BOCKAERT, J. (1982). Characteristics and Metabolism of α_1 adrenergic receptors in a nonfusing muscle cell line. *J. Biol. Chem.*, **257**, 875–879.
- PORZIG, H., BECKER, C. & REUTER, H. (1982). Competitive and non-competitive interactions between specific ligands and β -adrenoceptors in living cardiac cells. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **321**, 89–99.
- SCHWARZ, K.R., LANIER, S.M., CARTER, E.A., GRAHAM, R.M. & HOMCY, C.J. (1985). Transient high-affinity binding of agonist to α_1 -adrenergic receptors of intact liver cells. *FEBS Lett.*, **187**, 205–210.
- SHERMAN, W.R., LEAVITT, A.L., HONCHAR, M.P., HALLCHER, L.M. & PHILLIPS, B.E. (1981). Evidence that lithium alters phosphoinositide metabolism: Chronic administration elevates primarily D-myo-inositol-1-phosphate in cerebral cortex of the rat. *J. Neurochem.*

- 36, 1947–1951.
- SIBLEY, D.R. & LEFKOWITZ, R.J. (1985). Molecular mechanisms of receptor desensitization using the β -adrenergic receptor-coupled adenylate cyclase system as a model. *Nature*, **317**, 124–129.
- SLADECZEK, F., BOCKAERT, J. & MAUGER, J.-P. (1983). Differences between agonist and antagonist binding to α_1 -adrenergic receptors of intact and broken-cell preparations. *Mol. Pharmacol.*, **24**, 392–397.
- SLADECZEK, F., SCHMIDT, B.H., ALONSO, R., VIAN, L., TEP, A., YASUMOTO, T., CORY, R.N. & BOCKAERT, J. (1988a). New insights into maitotoxin action. *Eur. J. Biochem.*, **174**, 663–670.
- SLADECZEK, F., SCHMIDT, B.H., CORY, N.M., EL MOATASSIM, C., ALONSO, R., KIRK, K.L., KIRK, C.J., ROUOT, B. & BOCKAERT, J. (1988b). Complex interactions of α_1 -adrenoceptors in intact cells. *Br. J. Pharmacol.*, **95**, 1133–1140.
- STAEHELIN, M., SIMONS, P., JAEGGI, K. & WIGGER, N. (1983). CGP 12177: A hydrophilic β -adrenergic receptor radioligand reveals high affinity binding of agonists to intact cells. *J. Biol. Chem.*, **258**, 3496–3502.
- STEINMAN, R.M., MELLMAN, I.S., MÜLLER, W.A. & COHN, Z.A. (1983). Endocytosis and the recycling of plasma membrane. *J. Cell. Biol.*, **96**, 1–27.
- TOEWS, M.L. (1987). Comparison of agonist-induced changes in β - and α_1 -adrenergic receptors of DDT1 MF-2 Cells. *Mol. Pharmacol.*, **31**, 58–68.
- YARDEN, Y., RODRIGUEZ, H., WONG, S.K.-F., BRANDT, D.R., MAY, D.C., BURNIER, J., HARKINS, R.N., CHEN, E.Y., RAMACHANDRAN, J., ULLRICH, A. & ROSS, E.M. (1986). The avian β -adrenergic receptor: Primary structure and membrane topology. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 6795–6799.

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