

Transient high-affinity binding of agonists to α_1 -adrenergic receptors of intact liver cells

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Received 21 May 1985

At α_1 -adrenergic receptors in isolated rat liver parenchymal cells, (-)-epinephrine is potent in eliciting a maximal increase in glycogenolysis ($K_{act} = 24$ nM). This contrasts with a 100-fold lower affinity for the agonist at α_1 -adrenergic receptors of intact hepatocytes determined from equilibrium competition assays with the α_1 -adrenergic antagonist [³H]prazosin. We demonstrate here that agonists bind to α_1 -adrenergic receptors of intact liver cells initially with a markedly higher affinity than under equilibrium conditions. When incubations are performed for 15 s at 37°C, the affinity is more than 100-fold higher than that obtained in equilibrium (45 min) assays ($IC_{50} = 28 \pm 3$ vs 5300 ± 400 nM for (-)-epinephrine and 32 ± 3 vs 6100 ± 500 nM for (-)-norepinephrine). When incubations are performed at 4°C (150 min), high-affinity binding similar to that obtained in short-term incubations can also be demonstrated. In contrast, antagonists compete with similar affinities in 15 s and 45 min assays, and their dissociation constants are not affected by changes in the incubation temperature. These results indicate that agonists bind to native α_1 -adrenergic receptors transiently with high affinity. The conversion of receptors to a state of predominantly low affinity for agonists, which occurs rapidly and irreversibly with increasing incubation at 37°C, is inhibited at low incubation temperatures. It is suggested that the high-affinity configuration of the α_1 -adrenergic receptor for agonists observed in nonequilibrium experiments or at reduced incubation temperatures represents the physiologically relevant state of the α_1 -adrenergic receptor.

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| α_1 -Adrenergic receptor | Isolated hepatocyte | Glycogenolysis | Nonequilibrium binding | Conformational change |
| | | Receptor affinity state | | |

1. INTRODUCTION

Isolated rat hepatocytes have proved to be useful for the investigation of α_1 -adrenergic-receptor mediated effects [1–3]. Radioligand binding studies of adrenergic receptors conducted with intact cells, where the receptors remain in their native environment and attached to their effector systems, logically complement functional studies and should facilitate direct comparisons between functional assays and radioligand studies. However, such studies have revealed marked

discrepancies at β -adrenergic receptors, where the affinity of agonists is several orders of magnitude lower in competition assays with radiolabeled antagonists than that predicted from their relative potency in eliciting maximal increases in cAMP [4,5]. A similar discrepancy is present in α_1 -adrenergic receptors in isolated hepatocytes, where agonists are potent stimulators of glycogenolysis [6].

We characterized the binding properties of agonists at α_1 -adrenergic receptors of isolated hepatocytes under equilibrium and nonequilibrium conditions. Here we report on a phenomenon of transient high-affinity binding of agonists to α_1 -adrenergic receptors of intact liver cells.

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2. MATERIALS AND METHODS

2.1. *Preparation of hepatocytes*

Parenchymal liver cells were prepared by collagenase perfusion of livers from female Sprague-Dawley rats (180–240 g, Charles River Farm) maintained on standard Purina laboratory chow as described [7]. Cells were maintained in Dulbecco's minimal essential medium (MA Bioproducts, Walkersville, MD) at a concentration of 2×10^6 /ml at 37°C prior to use.

2.2. *Radioligand binding studies with intact hepatocytes*

For binding assays, cells were washed twice with ice-cold PBS (10 mM, Dulbecco's formula, pH 7.4) at $50 \times g$ for 5 min. In preliminary studies, several buffers [oxygenated Krebs-Ringer-bicarbonate (95% O₂/5% CO₂) or DMEM with Earl's salt solution] were employed and found to yield results identical to those obtained with PBS. Binding studies were performed in a total volume of 150 μ l with a cell concentration of 200000/test tube [100 μ l cell suspension, 25 μ l [³H]prazosin (0.4–0.7 nM), and 25 μ l of competing ligand, in triplicate]. (–)-Epinephrine and (–)-norepinephrine were dissolved in buffer containing 0.1 mM ascorbic acid. When incubations were carried out at 4°C, cells and competing ligands were precooled separately for 15 min before the binding reaction was started. For nonequilibrium studies, a total assay volume of 1 ml was employed. The binding reaction was terminated by washing tubes with 4×4 ml aliquots of PBS (25°C) onto 24 mm glass fiber filters (Schleicher and Schuell no.32), and unbound ligand was separated by vacuum filtration. Filters were placed in 10 ml scintillation fluid (Hydrofluor) and counted in a liquid scintillation spectrometer (Beckman) with a counting efficiency of 56%. Data obtained from equilibrium radioligand binding studies were analysed with the non-linear computer-assisted weighted least-squares curve-fitting procedure, LIGAND, which fits the untransformed data directly to possible curves derived from the law of mass action using Marquard's Algorithm [8]. Statistical analysis of 'goodness of fit' for several models of ligand-receptor interaction (binding to one or more binding sites) was performed as described by Munson and Rodbard [8]. For nonequilibrium binding ex-

periments, the IC₅₀ (the concentration of competing ligand that inhibited 50% of specific binding) and the percentage of high- and low-affinity sites for agonists were calculated with a 4-parameter-logistic expression ('twositehyp') [9].

2.3. *Chemicals*

[³H]Prazosin (80.9 Ci/mmol) was purchased from New England Nuclear. (–)-Epinephrine and (–)-norepinephrine were purchased from Sigma and prepared in buffer containing 0.1 mM ascorbic acid. Prazosin and phentolamine were gifts from Pfizer Corp., Groton, CT and Ciba-Geigy, Summit, NJ, respectively. All other reagents were purchased from Sigma (St. Louis, MO).

3. RESULTS

3.1. *Binding of [³H]prazosin to intact hepatocytes*

As previously shown [7], binding of the specific α_1 -adrenergic antagonist [³H]prazosin to intact isolated hepatocytes was saturable, reversible and of appropriate specificity [10]. Computer-assisted analysis of [³H]prazosin binding indicated interaction with a single class of sites ($B_{\max} = 95\,500 \pm 5500$ receptors per cell) with a dissociation constant of 233 ± 40 pM.

3.2. *Competition assays with agonists and antagonists in intact rat liver cells*

The affinity of agonists for α_1 -adrenergic receptors of intact hepatocytes was investigated both under equilibrium conditions and under nonequilibrium conditions, where the ligand-receptor interaction follows initial-velocity conditions ($[RL] \ll [R][L]$). Under the latter conditions, the IC₅₀ provides an estimate of the K_D for rapidly equilibrating ligands, as detailed in [10]. Competition experiments were carried out with incubation times ranging from 0.25 to 45 min. The concentration of radioligand used was between 1.5- and 3-times the K_D value. 'Specific' binding (binding inhibitable by 10^{-4} M (–)-epinephrine) was 50–70% at 0.25 min and 70–90% at 45 min. At 0.25 min, 5–10% of the binding that was observed after 45 min of incubation was present. When competition assays were performed for short incubation times (0.25 min), the resulting curves were biphasic and could be resolved into two different classes of binding sites (fig.1). (–)-

Norepinephrine competed with [3 H]prazosin for 76% of all binding sites with an IC_{50} of 32 ± 3 nM and for 24% with an IC_{50} of 6100 ± 500 nM. Virtually identical results were obtained with (-)-epinephrine as the competing ligand (28 ± 3 vs 5300 ± 400 nM, fig.2). With increasing incubation intervals, the competition curve for both agonists shifted progressively to the right (figs 1,2). The transition to predominant low-affinity binding occurred rapidly with a $t_{1/2}$ of approx. 1 min. At equilibrium (45 min incubation), inhibition curves were still biphasic; (-)-norepinephrine inhibited 79% of all [3 H]prazosin-binding sites with a dissociation constant (K_d) of 2000 ± 200 nM ($n = 5$) and the remaining 21% with a K_d of 15 ± 2 nM (fig.1). No further change in affinity occurred when incubations were continued for longer time periods. Similar proportions of high- and low-affinity sites were determined with (-)-epinephrine as competing ligand (fig.2).

The non-equilibrium approach was validated in studies with α -adrenergic antagonists. Phentolamine inhibited the binding of [3 H]prazosin with similar affinity at 15 s or 45 min. At

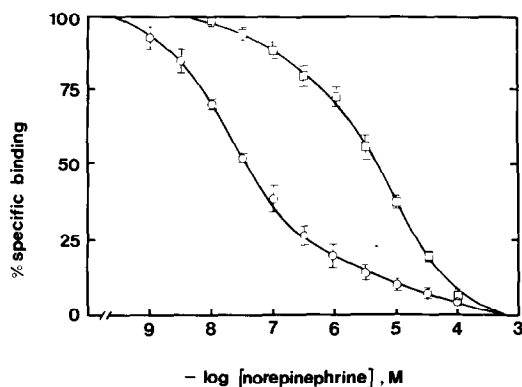


Fig.1. Inhibition of the binding of [3 H]prazosin to intact liver cells by (-)-norepinephrine under equilibrium and nonequilibrium conditions. Liver cells were incubated with [3 H]prazosin (0.4 nM) and the indicated concentrations of (-)-norepinephrine for 0.25 (\circ) or 45 (\square) min at 37°C. Specific binding at 0.25 min was 400–900 cpm (6–10% of that observed after 45 min of incubation). The results are expressed as the percentage of the radioligand bound at the indicated concentration of competing ligand. Points depicted are the means \pm SE from 3 experiments.

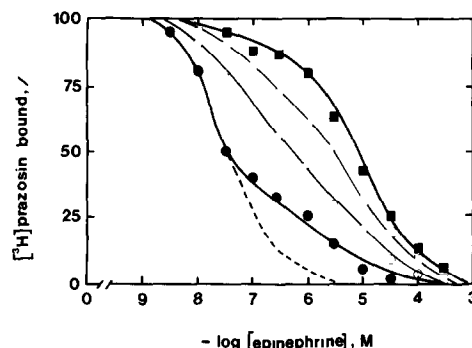


Fig.2. Inhibition of the binding of [3 H]prazosin to intact liver cells by (-)-epinephrine at increasing incubation times. Liver cells were incubated with [3 H]prazosin (0.4 nM) and the indicated concentrations of (-)-epinephrine for 0.25 (\bullet), 0.5 (\circ), 1.0 (\square) and 45 (\blacksquare) min at 37°C. Points depicted are from an individual experiment, which is representative of 5 similar experiments. Percentages of high-affinity sites were 70 (0.25 min), 52 (0.5 min), 60 (1 min) and 11% (45 min). The dashed line represents the predicted curve if all binding sites were in the high-affinity state.

equilibrium, the inhibition curve for this ligand was shifted slightly (up to 3-fold, as compared to 100–500-fold for agonists) to the right of that obtained in 15 s assays, depending on the concentration of [3 H]prazosin used [11] (fig.3). At all incubation times, competition curves with phentolamine were indicative of an interaction with a single class of binding site.

3.3. Functional studies in isolated hepatocytes

To determine the K_{act} for the α_1 -adrenergic receptor mediated response, the activation of glycogen-phosphorylase α in isolated hepatocytes was determined in isolated hepatocytes under similar conditions to those used for the radioligand studies. Activation of glycogen-phosphorylase α upon addition of (-)-epinephrine was rapid in onset and near maximal in 30 s. Maximal stimulation was 100% above basal activity of 15.1 ± 2.5 nmol [14 C]glucose incorporated into glycogen/min per mg protein. The K_{act} value in these studies for (-)-epinephrine was 24 nM, which is in agreement with that determined by others in these cells [12,13] and, as shown in figs 1 and 2, with the K_D estimates obtained in initial-velocity radioligand binding studies.

3.4. Effect of incubation temperature on the affinity of adrenergic agonists and antagonists

Previous studies have indicated that the decrease in affinity for β -adrenergic receptors of intact cells is temperature-dependent [10]. We therefore investigated the interactions of agonists and antagonists with α_1 -adrenergic receptors at reduced temperatures. Incubations were performed at 4°C for 150 min, a time period which proved to be sufficient to reach maximal binding. Under these conditions, (–)-epinephrine was approx. 100-fold more potent than at 37°C (fig.4A). The competition curves were best described by exclusive interaction of (–)-epinephrine with high-affinity sites ($K_D = 22$ nM, $n = 10$). This value is similar to that obtained in short-time incubation studies. In additional studies, the affinity of clonidine, a partial agonist which causes 30–50% of

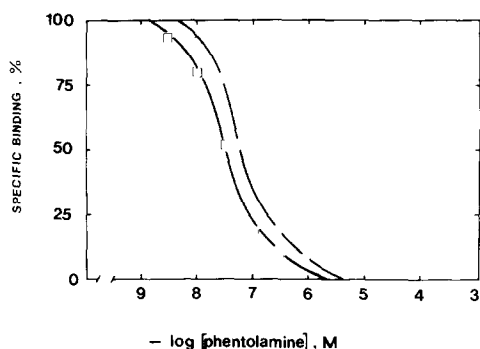


Fig.3. Inhibition of the binding of [3 H]prazosin to intact liver cells by phentolamine under equilibrium and nonequilibrium conditions. Liver cells were incubated with [3 H]prazosin (0.7 nM) and the indicated concentrations of phentolamine for 0.5 (\square) or 45 (\circ) min at 37°C. The results are expressed as percentage of the [3 H]prazosin bound at the indicated concentrations of competing ligand. Points depicted are from an individual experiment, which is representative of 3 similar experiments. With increasing incubation times, the competition curve for phentolamine shifts slightly to the right. The degree of the rightward shift was dependent on the concentration of radioligand used [11]. In most experiments, the rightward shift was only minimal, as concentrations of radioligand were used that were only slightly higher than the K_D . In this experiment, a concentration of radioligand was used that was 3-fold higher than the K_D , and the IC_{50} is approx. 3-fold lower at equilibrium conditions than that at 15 s.

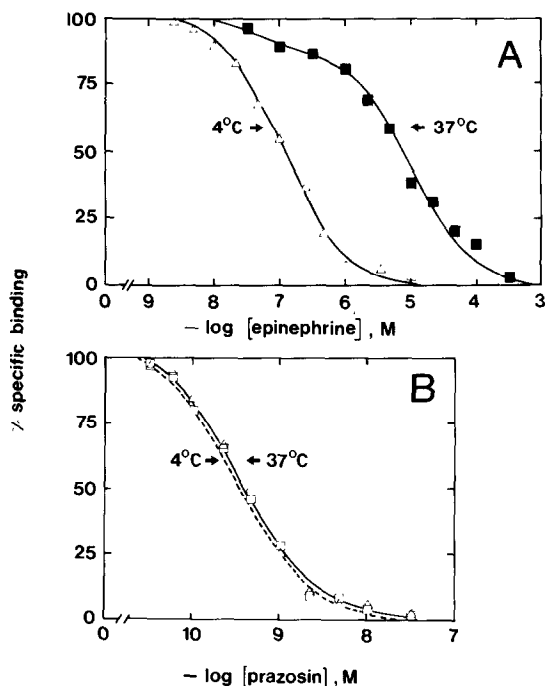


Fig.4. Effect of temperature on the affinity of (–)-epinephrine (A) and non-radioactive prazosin (B) for α_1 -adrenergic receptors of intact hepatocytes. (A) Isolated hepatocytes were incubated with [3 H]prazosin (0.7 nM) and increasing concentrations of (–)-epinephrine for 45 min at 37°C (\blacksquare) or 150 min at 4°C (\triangle). Points depicted are from an individual experiment, which is representative of 10 similar experiments. (B) Isolated hepatocytes were incubated with [3 H]prazosin (0.7 nM) and increasing concentrations of nonradioactive prazosin for 45 min at 37°C (\triangle --- \triangle) or 150 min at 4°C (\square --- \square). The results are expressed as percentage of the [3 H]prazosin bound at the indicated concentration of competing ligand. Points depicted are from an individual experiment, which is representative of 4 similar experiments.

epinephrine-induced stimulation of glycogenolysis, was also dependent on temperature, but increased only 10-fold at low incubation temperatures, as compared to 100-fold increases observed for full agonists (not shown). In contrast, the affinity of antagonists was not changed by incubation at reduced temperatures (fig.4B). This finding was confirmed by Scatchard plots of saturation studies where the affinity for [3 H]prazosin was found to be identical when incubations were performed at 37°C ($K_D = 233 \pm 40$ pM) or 4°C ($K_D = 260 \pm 56$ pM, $n = 8$).

4. DISCUSSION

Here we present evidence that agonists bind to α_1 -adrenergic receptors of rat liver cells initially with a markedly higher affinity than that demonstrated under equilibrium conditions. This high-affinity binding, which can also be demonstrated when incubations are performed at 4°C, is in good agreement with the K_{act} obtained in functional studies. In contrast, the dissociation constant for agonists in intact cells obtained under equilibrium conditions appears to be misrepresentative of the true affinity, as it reflects only the endpoint of a conversion of receptors to a predominant state of low affinity.

Our results are in complete agreement with findings previously obtained on β -adrenergic receptors in intact cells, where a marked discrepancy exists between the potency of agonists to increase cAMP (K_{act}) and their affinity constant calculated from equilibrium competition radioligand binding studies [4,10,14–16]. In all cell types examined, high-affinity binding of agonists could be demonstrated under initial velocity conditions, while few or no high-affinity sites could be detected at equilibrium [10,14–16]. Thus, the results of equilibrium studies in intact cells conducted at 37°C are not predictive of the affinity for agonists under physiological conditions, i.e. in the first seconds of the hormone-receptor interaction.

For β -adrenergic receptors, there is now good evidence to support the conclusion that those binding sites exhibiting high affinity for agonists represent ternary complexes of hormone, receptor and guanine-nucleotide-binding protein (G-protein) [17]. At these receptors in intact cells, the kinetics of the decrease in agonist affinity followed a similar time course to the induction of a refractory state [14]. It was thus suggested that the conversion to predominant low-affinity binding of agonists reflected an 'uncoupling' of the receptor from the adenylate cyclase system. As repeated stimulation with agonists does not lead to a diminution of α_1 -adrenergic receptor-mediated effects on glycogenolysis and to a 'downregulation' of receptor number in rat liver [18–20], such a mechanism fails to explain the observed decrease in agonist affinity in our experiments.

While the induction of a refractory state seems unlikely to be related to the phenomenon described

in this communication, we believe that the decrease in affinity most likely reflects a conformational change within the receptor molecule. This was previously suggested by Weiland et al. [21] based on the thermodynamic analysis of agonist binding. These studies showed that the binding of agonists is associated with a large decrease in enthalpy, while antagonist binding is almost exclusively entropy driven. It seems possible that this conformational change is involved in the information transfer across the cell membrane. Alternatively, it may represent a 'shut-off' mechanism to regulate adrenergic receptor-mediated effects independent of agonist off-rate.

The α_1 -adrenergic receptor is typically not 'coupled' to adenylate cyclase. Following the initial observation by Goodhart et al. [22], that binding to this receptor type can be modulated by guanine nucleotides, recent studies suggest that a novel G-protein may be involved in the signal transduction pathway initiated by α_1 -adrenergic stimulation [23]. However, there is as yet no biochemical evidence that those binding sites exhibiting a high affinity for agonists represent ternary complexes of hormone, receptor and G-protein.

A high affinity for agonists when incubations were performed at low incubation temperature was also reported for α_1 -adrenergic receptors in a smooth muscle cell line [24], as well as for β -adrenergic receptors of intact cells [10,25]. It remains to be clarified why the transition to predominant low-affinity binding is inhibited at low incubation temperatures. It is tempting to speculate that the constraints imposed by low temperatures, such as increased membrane rigidity may prevent an internal rearrangement of the receptor molecule itself.

In summary, our results demonstrate that agonists bind to native α_1 -adrenergic receptors of intact rat hepatocytes transiently with high affinity. Moreover, a conformational change of the agonist binding site, which seems to be fundamentally linked to the 'efficacy' of agonists, may be a common feature of all adrenergic receptor subtypes, independent of their different signal-transduction pathways.

ACKNOWLEDGEMENTS

These studies were supported in part by National Institute of Health Grants H1-19259 and NS-1919583, AHA Grant 83-1242 with funds from the Massachusetts Affiliate, and a grant from the R.J. Reynolds Company. R.M.G. and C.J.H. are Established Investigators, American Heart Association (grants AHA 80-148 and 82-240).

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