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Agonist-induced α_1-adrenergic receptor changes

Evidence for receptor sequestration

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Short-term receptor regulation by agonists is a well-known phenomenon for a number of receptors but little is known about the regulation of α_1-adrenergic receptors. In the present study we provide evidence of α_1-adrenergic receptor changes induced by agonists on DDT_1 MF-2 smooth muscle cells. The cells were preincubated with the agonist and receptor changes were investigated in the cells washed free of the agonist. On cells pretreated with norepinephrine the number of receptors recognized by [^{3}H]prazosin at 4°C was reduced by 38%. The receptors were not degraded as the number of sites was the same in control and norepinephrine-treated cells when binding was measured at 37°C. When binding was measured on fragmented membranes (at 4°C), the number of receptors was the same in control and norepinephrine-treated cells, suggesting that the disruption of cellular integrity might expose receptors which are probably sequestered after agonist treatment. We conclude that agonists induced rapid sequestration of receptors on intact DDT cells.

α_1-Adrenergic receptor; Receptor sequestration; Prazosin binding

1. INTRODUCTION

The interaction of a receptor with the specific agonist triggers a cascade of events that finally lead to the physiological response. At the same time the agonist induces changes in receptors so that, for a limited time, the receptor complex becomes less sensitive to subsequent stimulation (receptor desensitization). The changes that accompany (and probably sustain) desensitization have been well characterized for a number of receptors including β-adrenergic receptors [1,2]. However, the short-term regulation of α_1-receptors by agonists has been investigated little. Two previous papers reported that on intact BC3H-1 muscle cells [3] and rat liver cells [4] α_1-adrenergic receptors showed low affinity for agonists. The low-affinity form of the receptor was generated during the interaction of agonists with intact cells at 37°C [4]. This suggests an agonist-induced receptor change, but no further evidence supporting this possibility has been provided. In the present investigation of this possibility we obtained the first evidence of agonist-induced α_1-adrenergic receptor sequestration.

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Abbreviations: DDT cells, DDT_1 MF-2 smooth muscle cells; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; B_{max}, maximum receptor density; K_d, receptor dissociation constant

2. MATERIALS AND METHODS

2.1. *Cell culture*

DDT_1 MF-2 smooth muscle cells (DDT cells) were kindly provided by Dr M. Caron with the per-

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mission of Dr J.S. Norris (University of Arkansas). Cells were grown on 0.5–1 l suspension cultures under permanent gentle agitation. Growth medium was high-glucose DMEM (Gibco), supplemented with 10% fetal calf serum (Gibco). Cell viability exceeded 95% under all experimental conditions.

Since the number of receptors per cell was inversely related to the density of cells in suspension culture (unpublished), in some cases, to compare independent experiments, the data are presented as percentages of control values. In any case, to minimize differences, in the majority of experiments cells were used when the density in suspension culture was 2–4 × 10^5 cells/ml.

2.2. *Preincubation with norepinephrine*

DDT cells (5 × 10^6/ml) were incubated with 100 μM norepinephrine for 20 min at 37°C in DMEM containing 20 μg/ml each of superoxide dismutase and catalase (to prevent oxidation of norepinephrine [5]) and 1 μM (−)-propranolol (to exclude β-adrenergic receptor-mediated effects). After 20 min at 37°C the samples were placed in an ice-water bath, diluted to 50 ml with ice-chilled PBS and centrifuged at 200 × *g* for 10 min at 4°C. This washing procedure was repeated three more times, always at 4°C.

2.3. *α_1-Adrenergic receptor binding to intact DDT cells*

Cells suspended in incubation medium (DMEM containing 20 mM Hepes and 1 mg/ml bovine serum albumin, pH 7.4 at 25°C) were incubated with [^{3}H]prazosin in a final volume of 1 ml (2–5 × 10^5 cells/sample) for 45 min at 37°C, or 20–24 h at 4°C. Incubations were stopped by the addition of 10 ml ice-chilled PBS (Eurobio), rapid filtration through GF/C glass fibre filters (Whatman) and one additional 10 ml wash. Saturation experiments were performed using 5–8 concentrations of [^{3}H]prazosin (0.02–1.3 nM), each assayed in duplicate or triplicate. In some experiments we used a single saturating concentration of radioligand (0.4–0.6 nM assayed in triplicate or quadruplicate) which bound 95% of the total receptors. Non-specific binding, measured in the presence of 10 μM phentolamine, was less than 10% of the total binding at 2 × K_d.

2.4. *α_1-Adrenergic receptor binding on membranes*

DDT cells were homogenized with a glass-Teflon homogenizer for 5 min in incubation buffer (150 mM NaCl, 12 mM Hepes, pH 7.5 with NaOH) and this membrane preparation was used directly for binding. We avoided any washing because loss of receptors associated with light membrane fractions has been reported during conventional centrifugation [6]. Cell disruption was roughly 90% and apparently unbroken cells were not viable. All the binding procedures were the same as for intact cells except that the incubation medium was different (described above) and that glass fibre filters were soaked with 2% polyethyleneimine.

Proteins were determined by the method of Lowry et al. [7].

Saturation binding isotherms were analyzed by a non-linear regression computer program [8]. Data are presented as the mean ± SE. B_{max} values are expressed as sites/cell in experiments on intact cells, or as fmol/mg protein in experiments on membranes; K_d are given in nM.

2.5. *Chemicals*

Sources of chemicals: (−)-propranolol, gift from Imperial Chemical Industries (England); superoxide dismutase, gift from Zambeletti (Baranzate, Italy); [^{3}H]prazosin (60 Ci/mmol), Amersham International; phentolamine, Ciba Geigy (Origgio, Italy); (−)-norepinephrine, epinephrine and catalase, Sigma (St. Louis, MO).

3. RESULTS

On intact DDT cells [^{3}H]prazosin only bound to a single class of sites. Specific binding was saturable and of high affinity (K_d 0.094 ± 0.011 nM at 37°C; 0.040 ± 0.006 nM at 4°C; n = 5). In parallel experiments [^{3}H]prazosin bound to the same number of receptors at 37 and 4°C (48200 ± 11100 and 47800 ± 11500 sites/cell respectively; n = 3).

On intact DDT cells pretreated with 100 μM norepinephrine at 37°C for 20 min, the number of α_1-adrenergic receptors (measured at 4°C) was reduced by 38 ± 3% (n = 10) whereas when [^{3}H]prazosin binding was determined at 37°C for 45 min, the number of α_1-receptors was similar in

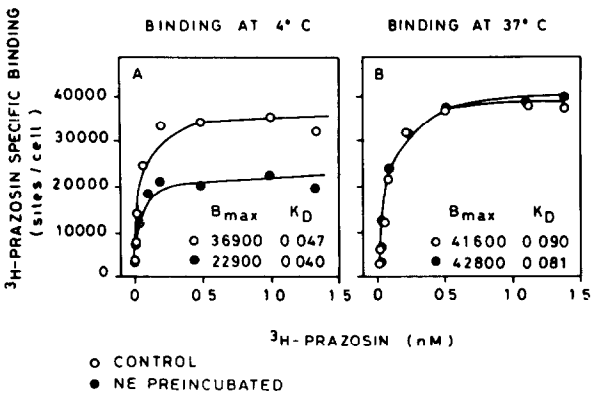

Fig.1. Effect of norepinephrine treatment on [^{3}H]prazosin binding to intact DDT cells. The cells were incubated without (control) or with 100 μM norepinephrine (NE) for 20 min at 37°C and then extensively washed. [^{3}H]Prazosin binding on intact cells was measured for 20 h at 4°C (A) or 45 min at 37°C (B). The experiment shown is representative of three.

control and norepinephrine-treated cells (fig.1). The induction of this receptor decrease had a $t_{1/2}$ of about 3 min, reaching maximal effect after 10–15 min with no further decrease up to 60 min (fig.2). Over this time course the sites recognized by [^{3}H]prazosin at 37°C were unchanged by norepinephrine treatment (fig.2). Pretreatment with epinephrine (100 μM) induced the same reduction of sites recognized by [^{3}H]prazosin at 4°C (B_{max} 53100 and 32700 sites/cell, respectively for control and treated DDT cells) without any change in the K_d (0.037 and 0.036 nM).

The recovery of this effect was also investigated. DDT cells were preincubated with norepinephrine (20 min at 37°C), extensively washed and then incubated in agonist-free medium for various times at 37°C, after which [^{3}H]prazosin binding was assayed at 4°C for 20 h (fig.3). Complete recovery from the sequestered state required more than 12 h of agonist-free incubation, while virtually no recovery was seen in the first hour.

The reduction of receptors observed with [^{3}H]prazosin binding at 4°C on intact cells may have two explanations: (i) norepinephrine pretreatment induces a conformational change in some receptors so that they are no longer recognized by [^{3}H]prazosin in the cold; (ii) norepinephrine treatment induces sequestration of receptors in the cellular environment which is inaccessible to the

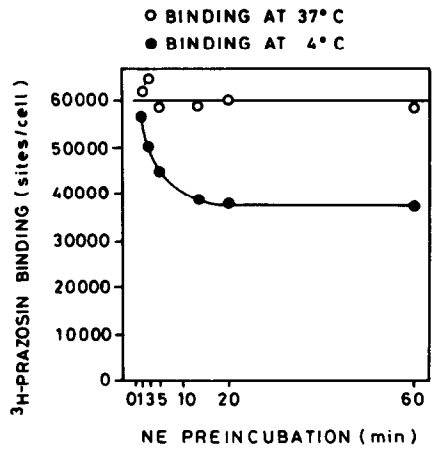

Fig.2. Time course of α_1-adrenergic receptor sequestration during norepinephrine treatment. DDT cells were incubated with 100 μM norepinephrine (NE) for the indicated times at 37°C. Then the cells were chilled, extensively washed and resuspended in cold DMEM. Cells from each time point were assayed in parallel for [^{3}H]prazosin (0.4 nM) binding at 37°C (45 min) or at 4°C (20 h). The experiment shown is representative of two.

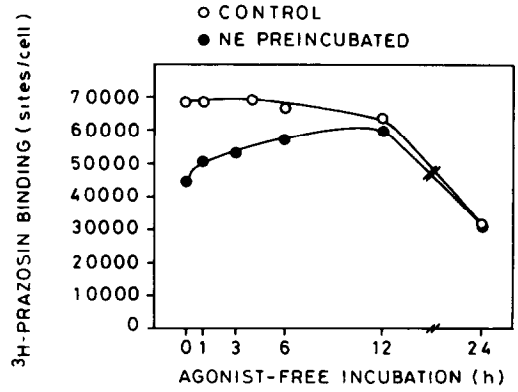

Fig.3. Time course of α_1-adrenergic receptor recovery from the sequestered state. DDT cells were incubated without (control) or with 100 μM norepinephrine (NE) for 20 min at 37°C and extensively washed. The cells were resuspended in serum-free DMEM and kept at 37°C for various times. [^{3}H]Prazosin binding was assayed at 4°C for 20 h. A single saturating concentration of [^{3}H]prazosin (0.5 nM) was used. The experiment shown is representative of three.

ligand at 4°C. We therefore measured the binding of [^{3}H]prazosin in fragmented cells on the assumption that the disruption of cellular integrity might expose the presumed sequestered sites. Indeed, the number of receptors was similar on fragmented membranes from control and norepinephrine treated cells (fig.4). In parallel experiments with norepinephrine-treated cells the binding of [^{3}H]prazosin (at 4°C) amounted to 58 ± 3% of that on intact cells but up to 88 ± 11% of the relative control values at 37°C when the cells were broken before performing the binding assay. Binding to the controls (intact cells and cells broken before performing binding assay) at 4°C was 92 ± 5 and 99 ± 5% respectively, while binding on norepinephrine-preincubated DDT cells was 98 ± 5 and 99 ± 7% respectively, the percentages being referred to relative control values at 37°C. The binding at 4°C on norepinephrine-preincubated intact cells was the only one significantly different from all other values (Duncan's test, $P < 0.01$).

The binding of [^{3}H]prazosin (at 4°C) on intact DDT cells remained the same when incubation was continued for 20, 48 and 72 h on control (respectively 63700, 62500 and 63000 sites/cell) and norepinephrine-treated cells (respectively 49600, 50700 and 48800 sites/cell). This means that a reduced equilibration rate for [^{3}H]prazosin cannot account for the decreased binding on norepinephrine-treated cells. α_1-Adrenergic receptors were not reduced in cells exposed to norepinephrine at 4°C. DDT cells were incubated without (control) or with norepinephrine (100 μM) for 2 h at 4°C and then washed. The binding of [^{3}H]prazosin (measured for 20 h at 4°C) was similar in control and norepinephrine-treated cells (respectively 42000 and 42100 sites/cell). This also proves that norepinephrine is completely removed by the washing procedure used.

To test whether incubation for 2 h at 4°C allowed the interaction of norepinephrine with the receptors, DDT cells were incubated with [^{3}H]prazosin (0.5 nM) in the absence and presence of norepinephrine (100 μM) for 2 h at 4°C, and then filtered. Approx. 90% of the receptors were occupied by norepinephrine (22200 and 2800 sites/cell respectively in the absence and presence of norepinephrine), suggesting a wide interaction of the agonist with the receptors.

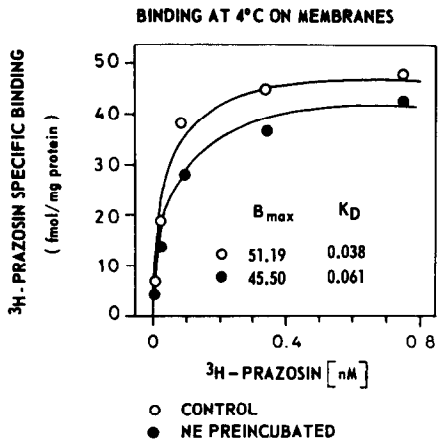

Fig.4. Effect of norepinephrine treatment on [^{3}H]prazosin binding to fragmented membranes. DDT cells were incubated without (control) or with 100 μM norepinephrine (NE) for 20 min at 37°C and extensively washed. Then the cells were fragmented and [^{3}H]prazosin binding assayed for 20 h at 4°C. The experiment shown is representative of two.

4. DISCUSSION

We present evidence that the interaction of agonists with α_1-adrenergic receptors on intact DDT cells at 37°C induces rapid changes in the receptors: in cells pretreated with agonist, approx. 40% of the receptors became inaccessible for [^{3}H]prazosin binding at 4°C. To the best of our knowledge this is the first evidence of sequestration of α_1-adrenergic receptors.

On DDT cells pretreated with norepinephrine the number of receptors recognized by [^{3}H]prazosin at 4°C was reduced by 38%. The receptors were not degraded as the number of sites was the same in control and norepinephrine-treated cells when binding was measured at 37°C. Virtually all the receptors could be recognized by [^{3}H]prazosin even at 4°C, when the control and norepinephrine-treated cells were broken and binding was measured on fragmented membranes.

These findings are consistent with the hypothesis that agonists are able to induce sequestration of α_1-adrenergic receptors: after exposure to norepinephrine at 37°C for a few minutes 38% of the receptors are removed from the cell surface and sequestered in an environment which in intact cells

is not accessible to [^{3}H]prazosin at 4°C. However, both surface and sequestered receptors were recognized by [^{3}H]prazosin at 37°C. The rigidity of biological membranes induced at low temperature may account for the different accessibility of [^{3}H]prazosin to the cellular compartment where receptors are sequestered. An alternative is that the sequestered receptors return to the cell surface during the 45 min of binding incubation at 37°C. However, this cannot be the case, since no recovery of receptor sequestration was observed up to 2 h of incubation at 37°C in agonist-free medium.

Sequestration of α_1-adrenergic receptors seems to be related to desensitization of α_1-mediated functional response. In fact in DDT cells pretreated with norepinephrine (100 μM), norepinephrine-stimulated phosphatidylinositol turnover was reduced. The time courses of reduction of surface receptors and decrease in stimulation of phosphatidylinositol turnover were similar (Leeb-Lundberg et al., in preparation).

Previous studies reported a reduced number of α_1-adrenergic receptors in membranes from smooth muscle and BC3H-1 cells preincubated for several hours with norepinephrine and epinephrine [9–11]. This effect, referred to as receptor down-regulation, must be taken as being distinct from the phenomenon of sequestration described here. Down-regulation occurs after long-term exposure to an agonist and involves receptor degradation resulting in a reduction of the total number of sites. By contrast, receptor sequestration occurs after a few minutes exposure to an agonist and involves receptor redistribution in a different cellular compartment without any change in their total number. Receptor sequestration appears to be the rapid mechanism of desensitization to acute hyperstimulation while down-regulation is the adaptive response to chronic exposure to agonists.

In conclusion our findings provide evidence of agonist-induced rapid sequestration of α_1-adrenergic receptors into cells.

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