SELECTIVE IDENTIFICATION OF 'TRUE' β-ADRENERGIC RECEPTORS IN THE PLASMA MEMBRANES OF RAT ADIPOCYTES

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1. Introduction

In recent years, several groups have reported in vitro binding of [^{3}H]catecholamines to plasma membranes from a variety of tissues [1–10]. These reports have described some binding properties which are commonly characteristic of hormone–receptor interaction (i.e. saturability, time- and temperature-dependence, displacement of bound hormone by unlabelled compounds and similar concentration ranges for the binding affinity and the cyclase activation).

However, these well-documented results are impaired by the discrepancy between the inhibition of the catecholamine-activated cyclase by β-blockers and the poor or non-existant inhibition of the hormone-binding by such compounds. Furthermore it appears that the number of true β-adrenergic receptors linked to the adenylate cyclase activation is much smaller than the total number of catecholamine binding sites [11]. The non-specific sites are either linked to a hypothetical 'catechol' binding moiety [9] or possibly to catechol-*o*-methyltransferase [11,12]. Therefore, no definitive conclusion has been reached regarding the true nature of the β-adrenergic receptor connected to the adenylate cyclase system.

Thus in order to identify the real receptor, it appears necessary to suppress, in an artificial manner, the binding related to the catechol moiety without modifying the adenylate cyclase activation.

In the present report, we have studied the binding of catecholamines to plasma membranes prepared from rat adipocytes, and have shown that the non-specific binding of the catechol moiety to these membranes is markedly decreased in the presence of 1 mM EGTA* or 0.5 mM pyrocatechol. Under these conditions, activation of adenylate cyclase by norepinephrine is not affected, while the binding of [^{3}H]norepinephrine is drastically reduced. The 'residual binding' of norepinephrine may be displaced by (−)propranolol and is sensitive to phospholipase A; furthermore, it can subsequently be related to the proportion of 'true' β-adreno-receptor binding sites.

2. Methods

Plasma membranes were prepared from adipocytes isolated from rat epididymal fat pads as previously described [13] and were stored under liquid nitrogen until use. Protein concentration was measured by the method of Lowry et al., [14] using bovine serum albumine (Fraction V Pentex) as standard.

Adenylate cyclase activity was determined by a modification of the method of Krishna et al., [15]. The incubation mixture (50 μl) contained 25 mM Tris–HCl buffer, pH 7.6, 5 mM $MgCl_2$, 0.4 mM [α.^{32}P] ATP (C.E.A. Saclay, France), 1 mM dithiothreitol, 0.1% (w/v) bovine serum albumin, an ATP regenerating system consisting of 10 mM phosphocreatinine and 0.1 mg/ml creatine kinase (Calbiochem, 100 units per mg of protein). The reaction was started by addition of the membrane enzyme. After incubating for 10 minutes at 37°C, 0.1 ml of a 'stopping' solution containing 40 mM ATP, 10 mM [^{3}H]cyclic AMP (20–30 × 10^3 cpm), 20 mM EDTA, and 50 mM Tris–HCl

* *Abbreviations*: EGTA, ethyleneglycol - bis (β-amino ethyl ether) *N, N'*-tetraacetic acid; EDTA, ethylenediamine tetraacetic acid (disodium salt).

(pH 7.4) were added. Cyclic AMP was isolated by chromatography on neutral alumina [16].

Binding assays of (–) [^{3}H]norepinephrine (specific activity 10–14 Ci/mmol from Radiochemical Center, Amersham) were performed at 37°C for 6 min under the conditions where adenylate cyclase activity was effective. The incubation mixture (120 μl) contained: 5×10^{-7} M [^{3}H]norepinephrine, 0.4 mM ATP, 5 mM $MgCl_2$, 1 mM dithiothreitol, 25 mM Tris–HCl (pH 7.4), and 70–90 μg of membrane protein. Membrane-bound radioactivity was collected on nitrocellulose filters (BA 85, Carl Schleicher Schüll), after washing twice with 5 ml of 5 mM Tris–HCl pH 7.4. Filters were previously preincubated in a large excess of a cold norepinephrine solution in order to decrease non-specific binding associated with the filter (blank value). Filters were dissolved by shaking in 10 ml of Unisolve (Koch Light) and counted for radioactivity.

3. Results

In order to overcome the problem of the specificity of norepinephrine binding, we have used two independent methods:

1) EGTA, a specific Ca^{2+} chelator, suppressed about 96% of the total catecholamine binding at 1 mM concentration (table 1). Under these conditions, the norepinephrine-enhanced adenylate cyclase activation was unaffected in spite of a faint modification of basal activity. Therefore a maximum of 4% of the total norephinephrine binding could be coupled to the adenylate cyclase system in fat cell membranes.

2) The catechol compound, pyrocatechol, competed strongly by a separate pathway with the [^{3}H]norepinephrine binding sites related to the catechol moiety. In contrast, no effect was seen on norepinephrine-induced adenylate cyclase activation (table 2A). Addition of pyrocatechol achieved about complete occupancy (98%) of the catecholamine sites. Thus, the residual binding was about 2% of the total binding sites determined in the absence of pyrocatechol (table 2A).

(–)Propranolol, a strong β antagonist of the norepinephrine-enhanced adenylate cyclase activity (90% antagonism at 10^{-6} M), displaced 50% of the residual binding at the same molarity. Similar data were obtained with ephedrine, a sympathomimetic non-phenolic amine (table 2B). Therefore, these antagonists of norepinephrine displaced a large part of the residual binding whereas they did not inhibit norepinephrine binding due to the catechol moiety (table 2A).

3) As shown in fig. 1, the treatment of membranes with a highly purified Ca^{2+}-dependent phospholipase A resulted in a selective loss of the norepinephrine response, whereas the stimulation of the enzyme response to fluoride was maintained. Addition of EGTA, prior to that of phospholipase A, antagonized the inhibitory effect of the latter on the stimulation of adenylate cyclase by norepinephrine and fluoride ion.

Table 1
Effect of EGTA on the adenylate cyclase activity of fat cell membranes and on [^{3}H]norepinephrine binding

	adenylate cyclase activity (pmol/min/mg prot. ± SE)		[^{3}H]norepinephrine binding (pmol/mg/prot. ± SE)
	Basal	(–)norepinephrine (10^{-4} M)	
Absence of EGTA	52 ± 3	213 ± 10	16.6 ± 2.0
Presence of EGTA 1 mM	38 ± 2	154 ± 8	0.8 ± 0.2

Adenylate cyclase assays were performed with 30 μg of membrane protein, while norepinephrine binding was determined with 73 μg of membrane protein in the presence of 5×10^{-7} M [^{3}H]norepinephrine (for details see Methods).

Table 2
Effect of pyrocatechol, (−)ephedrine and (−)propranolol on the norepinephrine-stimulated adenylate cyclase activity and on [^{3}H]norepinephrine binding to fat cell membranes

	Addition	[^{3}H]norepinephrine binding (pmol bound/mg prot. ± SE)	% inhibition of norepinephrine stimulated adenylate cyclase
	No addition	25.8 ± 1.9	0*
	(−) Ephedrine 5×10^{-4} M	25 ± 2	70
A	(−) Propranolol 10^{-6} M	26 ± 1.5	90
	Pyrocatechol 5×10^{-4} M	0.6 ± 0.08	0
	Pyrocatechol 5×10^{-4} M + (−) Ephedrine 10^{-4} M	0.4 ± 0.17	
B	+ (−) Ephedrine 5×10^{-4} M	0.28 ± 0.015	
	+ (-) Propranolol 10^{-6} M	0.3 ± 0.09	

For binding studies, the concentration of [^{3}H]norepinephrine was 5×10^{-7} M; 80 μg of membrane protein was used. Adenylate cyclase assays were performed with 12 μg of membrane protein in the presence of 5×10^{-6} M (−)norepinephrine as described in Methods.
* Stimulation in the presence of 5×10^{-6} M norepinephrine was 4-fold greater than the basal activity. All values were corrected for the intrinsequal effect of each compound on the basal activity.

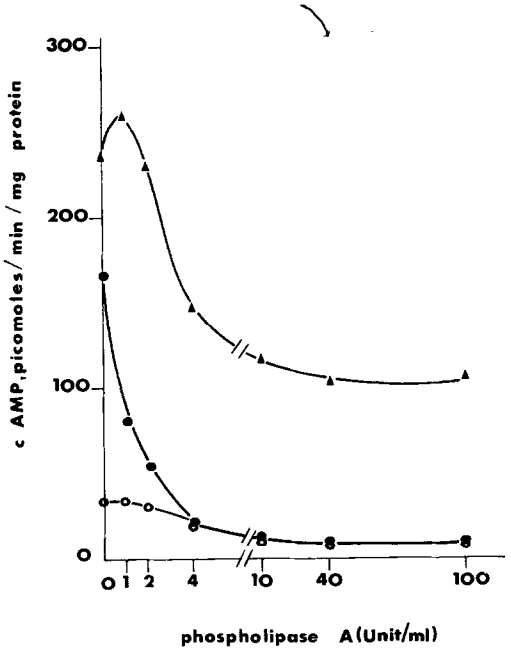

Fig. 1. Fat cell membranes (210 μg of protein) were incubated at 37°C for 5 min in 1 mM $CaCl_2$, 25 mM Tris–HCl, pH 7.6 (60 μl final vol) and with the indicated concentration of phospholipase A (from bee venom, Sigma, activity 1230 units/mg protein). Digestion was stopped by addition of 10 μl of 14 mM EGTA buffered with Tris at pH 7.6 and 10 μl aliquots (30 μg of membrane protein) were assayed for adenylate cyclase activity as described in Methods, in the absence (o–o–o), basal, and in the presence of 10^{-4} M (−)norepinephrine (•–•–•) or 10 mM F^- (▲–▲–▲).

The [^{3}H]norepinephrine binding assay determined with phospholipase A-treated membranes in the presence of EGTA, (which both blocked the enzymic digestion and suppressed the binding related to the catechol moiety) showed a 55% decrease in the residual binding (table 2).

Table 3
Effect of phospholipase A treatment on [^{3}H]norepinephrine 'residual' binding to fat cell membranes

	[^{3}H]norepinephrine binding (pmol/mg/prot. ± SE)
Control	0.77 ± 0.13
Phospholipase A treated* membranes	0.43 ± 0.10

Fat cell membranes (73 μg of protein) were incubated with phospholipase A (5 units per ml) for 5 min at 37°C under conditions similar to those described in the legend to fig. 1; then, EGTA was added so that its concentration was 1 mM final in binding assays, which were performed under the experimental conditions described in Methods (EGTA solution was buffered at pH 7.4).
* Preliminary experiments have shown that the total [^{3}H]norepinephrine binding measured in the absence of EGTA was not affected by phospholipase A treatment.

4. Discussion

The principal problem involved in studies of the interactions of catecholamines with their receptors is the lack of specificity of the binding of the catechol moiety. In the present report, we have demonstrated that this difficulty can be obviated by the use of either the catechol compound pyrocatechol or the chelating agent EGTA. Each of these compounds separately effected a decrease of 96–98% in the toal catechol binding to fat cell membranes, without any modification of the norepinephrine-induced adenylate cyclase stimulation. In this regard, Lacombe and Hanoune [17] have recently reported a decrease of 50% in the total amount of epinephrine bound by rat liver plasma membranes in the presence of EDTA. Under the present conditions, it appears that a maximum of 4% of the total norepinephrine binding sites are implicated in the mechanism of action of biogenic amines.

Propranolol is well established as an inhibitor of the lipolytic effect of epinephrine in fat cells [18] and of the epinephrine-induced stimulation of the adenylate cyclase system in white adipose tissue [19]. We have recently established that the inhibition of adenylate cyclase by propranolol is strictly competitive (J. Pairault, unpublished data). Therefore, it appears likely that this ligand competes for the 'true' β-adrenoreceptor associated with the adenylate cyclase system.

Until now, previous reports have failed to demonstrate the competition of propranolol for catecholamines-binding sites at the minimal concentration required to observe a 100% antagonism of the biological effect of catecholamines. We have shown that a fraction of the binding which is not displaced by pyrocatechol is decreased by about 50% by (−)ephedrine or (−)propranolol. The concentration of the compounds was consistent with their inhibitory effect on the stimulation of adenylate cyclase by norepinephrine. Thus, we assume that the authentic β-adrenoreceptors could represent 1 to 2% of the total catecholamine binding sites; therefore, at [^{3}H]norepinephrine concentration of 10^{-7} M, the number of the 'true' sites is equivalent to 0.3–0.35 pmol of norepinephrine bound per milligramm of protein. Using equilibrium dialysis, Levitsky et al. [20] have recently reported comparable data for the specific binding of [^{3}H]isoproterenol to turkey erythrocyte ghosts.

Furthermore, we have shown that the treatment of fat cell membranes by phospholipase A abolished the stimulation of adenylate cyclase by norepinephrine and in addition, decreased the residual 'binding' to an extent similar to that observed with ephedrine and propranolol. These observations provide evidence for the phospholipid nature of the authentic β-adrenoreceptors, and are consistent with studies in other tissues which have suggested that phospholipids are important in hormone-sensitive adenylate cyclase systems [21,23].

As the catechol structure and the ethanolamine side chain are of critical importance for the maximal expression of the β-adrenergic function [8,9], it is clear that 'true' β-adrenoreceptors contain both 'catechol' and stereospecific 'ethanolamine' [24] binding sites which are each associated with the adenylate cyclase system.

Our present results suggest that as long as the 'ethanolamine' site is occupied by an antagonist with an ethanolamine side chain, then the attachement of norepinephrine to the 'true' catechol moiety is prevented. Therefore, we propose the existence of a positive interaction between the two types of sites suggested by the structure of the catecholamines.

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