SOLUBILIZATION OF EPINEPHRINE-SPECIFIC α_2-ADRENERGIC RECEPTORS FROM ADRENOCORTICAL CARCINOMA

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1. Introduction

Normal isolated adrenal cells [1,2] are markedly and specifically stimulated by a singular polypeptide hormone, ACTH, in the production of corticosterone, indicating that these cells contain only one type of plasma membrane receptors that are coupled to the steroid metabolism. Adrenocortical carcinoma 494 [3] cells [4], in contrast, possess two additional epinephrine-binding receptors, α-adrenergic [5] and β-adrenergic [6]. While the biological function of these ectopic catecholamine-sensitive receptors is unknown, there is evidence that β-adrenergic receptors are coupled to adenylate cyclase [7] and α-adrenergic receptors to guanylate cyclase [8]. Thus the model system of neoplastic adrenal cells not only provides the opportunity to assess the relationship of these epinephrine-sensitive receptors in the endocrine control of neoplasia, it also enables the studies designed to elucidate the molecular mechanism of their hormonal regulation and transduction of the biological signal. Such studies could be greatly facilitated if these individual receptors could be isolated and purified. CHAPS, a zwitterionic detergent, has been originally used for the solubilization of opiate receptors [9]. Here, this detergent was successfully utilized in the selective solubilization of α-receptors. The results indicate that these receptors are of exclusively α_2-subtype. While the only biological function of α_2-receptors has been ascribed to the inhibition of adenylate cyclase [10,11], these results together with [8], indicate that another major function of these receptors may be the mediation of epinephrine-induced transmembrane signal via the activation of guanylate cyclase. Such a hormonal pathway would rationalize the mediatory role of cyclic GMP in the transduction of membrane signal.

Abbreviations: [^{3}H]DHE, [^{3}H]dihydroergocryptin; [^{3}H]-DHA, [^{3}H]dihydroalprenolol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate

2. Materials and methods

Adrenocortical carcinoma 494, a spontaneously occurring tumor discovered in [3] and maintained in our laboratory [4] was used for the membrane preparation and binding studies.

[^{3}H]DHE (spec. act. 39.9 Ci/mmol), and [^{3}H]-DHA (spec. act. 43.0 Ci/mmol) and ^{125}I-ACTH (spec. act. 30 μCi/μg) were obtained from New England Nuclear; (−)-epinephrine bitartrate, (−)-norepinephrine hydrochloride, yohimbine hydrochloride, ergotamine tartrate, α-ergocryptine, (−)-isoproterenol hydrochloride, and (±)-propranolol hydrochloride were from Sigma. $ACTH_{1-39}$ was from USP Corticotropin Reference Standard (Bethesda MA); phentolamine hydrochloride, prazosin hydrochloride and QUSO G-32 were gifts from Ciba-Geigy, Pfizer Inc. and Philadelphia Quartz Co., respectively. PD-10 Columns were purchased from Pharmacia Fine Chemicals.

2.1. *Membrane preparation*

Adrenocortical carcinoma membranes were prepared as in [12] with minor modifications. The fresh tumor tissue was collected into 10 mM Tris (pH 7.5) maintained on ice. Necrotic cells were removed, surrounding fibrous tissues were dissected away, and viable tissue was homogenized in 6 vol. ice cold buffer (0.25 M sucrose, 1 mM $MgCl_2$, 5 mM Tris–HCl (pH 7.5) for 4 × 30 s periods in a Brinkman Polytron

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at a setting of 6. After filtration through a double layer of gauze, the homogenate was centrifuged at 400 × *g* for 10 min at 4°C and the supernatant recentrifuged at 28 000 × *g* for 10 min at 4°C. The resulting pellet was washed twice in ice cold incubation buffer (10 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.5)) by resuspension and centrifugation at 28 000 × *g* for 10 min. The final pellet resuspended in incubation buffer was used for the binding assays. Protein was determined by the Bradford method using bovine serum albumin as a standard [13].

2.2. *Solubilization of receptors*

Adrenocortical carcinoma membranes were solubilized as in [9]. To the membrane suspension (~4 mg/ml) was added 50 μg/ml lima bean trypsin inhibitor, 0.01% dimethyl sulfoxide and 10 mM CHAPS (final conc.). This was stirred vigorously on ice for 1 h, then centrifuged at 105 000 × *g* for 60 min. The clear, slightly yellow supernatant was used for the binding studies.

2.3. *Binding assays*

2.3.1. Membranes

The binding assays were performed as indicated below unless otherwise stated. [^{3}H]DHE (8 nM) and adrenocortical carcinoma membranes (~400 μg/assay) were incubated at 37°C for 20 min in a total volume of 1.0 ml incubation buffer (10 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.5)). Incubation was terminated by diluting the incubation mixture with 5 ml buffer followed by immediate filtration through Whatman GF/A glass fiber filters which were washed with 4 × 5 ml incubation buffer. This procedure significantly reduced the non-specific binding of the ligand. After drying, filters were counted for radioactivity in Omniflor/toluene scintillation mixture. Non-specific binding was measured in the presence of 0.1 mM phentolamine, a potent α-adrenergic antagonist.

2.3.2. Solubilized fractions

Aliquots of the solubilized receptors were incubated in 1.0 ml final vol. for 20 min at 37°C in a mixture containing 50 mM Tris, 10 mM $MgCl_2$ (pH 7.5) and 8 nM [^{3}H]DHE. The reaction was stopped by adding 1.5 ml 10 mM Tris and 0.32 M sucrose (pH 7.5). After cooling on ice, the mixture was applied to a Sephadex G-25 column (PD-10), pre-equilibrated with 0.32 M sucrose, 10 mM Tris–HCl (pH 7.5). The initial 2.5 ml effluent was discarded. The high M_r fraction, eluted in the next 3.5 ml was collected and counted for radioactivity with 12 ml Scintiverse. Non-specifically bound radioactivity was determined in the presence of 0.1 mM phentolamine in the incubation mixture. All the experiments were done in triplicate and repeated at least 2 times. The specific binding was between 15–30% of the total counts bound.

3. Results

The zwitterionic detergent CHAPS is very selective in solubilizing α-adrenergic receptors. Adrenocortical carcinoma tissue contains α- and β-adrenergic as well as ACTH receptors. When binding studies were done for all the 3 receptors, it was found that only α-adrenergic receptors were solubilized (table 1). The recovery of protein in the solubilized fraction was ~30–45% with the specific activity of [^{3}H]DHE binding almost the same as that for the membrane receptors. The solubilized receptors exhibited identical binding characteristics as the membrane receptors. Fig.1A shows the binding of various concentrations of [^{3}H]DHE to the solubilized receptors. The saturation curve revealed

Table 1
Presence of adrenergic and ACTH receptors in adrenocortical carcinoma particulate and solubilized fractions

	Particulate	Solubilized
	(fmol/mg protein)	
α-Adrenergic receptors [^{3}H]DHE binding	50.7 ± 7.2	62.8 ± 1.9
β-Adrenergic receptors [^{3}H]DHA binding	29.7 ± 3.0	Non-detectable
ACTH receptors ^{125}I-ACTH binding	43.0 ± 3.2	Non-detectable

α- and β-Adrenergic receptors were quantitated both in the membrane and solubilized fractions as in section 2. The procedure for β-adrenergic receptor binding was the same as that of α-adrenergic receptors except the radioligand used was [^{3}H]DHA and non-specific binding was measured in the presence of 0.1 mM isoproterenol. ACTH binding was done on ice for 10 min using ^{125}I-ACTH and non-specific binding was measured in the presence of 1000-fold excess of $ACTH_{1-39}$. The total assay volume was 0.5 ml and the reaction was stopped by diluting the reaction mixture to 1.5 ml with incubation buffer (50 mM Tris–HCl (pH 7.5)). To this was added 10 mg QUSO and let stand for 10 min on ice. The bound ^{125}I-ACTH was separated from the free by centrifugation and the supernatant was counted for radioactivity in a γ-counter

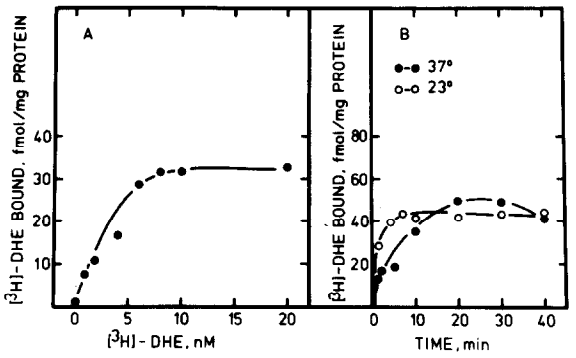

Fig.1. (A) Specific binding of [^{3}H]DHE to solubilized receptors as a function of [^{3}H]DHE concentration. The solubilized receptors were incubated at 37°C for 20 min with various concentrations of [^{3}H]DHE and the reaction was stopped by diluting the incubation mixture with buffer (10 mM Tris, 0.32 M sucrose (pH 7.5)) and passing through a PD-10 column; 3.5 ml column effluent was collected and counted for radioactivity after discarding the initial 2.5 ml. Non-specific binding was measured in the presence of 0.1 mM phentolamine–HCl. Experiments were repeated at least 3 times in triplicate and the results are representative of 1 expt. (B) [^{3}H]DHE binding at 37°C and 23°C with solubilized receptors as a function of time. Incubations were done for various time intervals at 37°C and 23°C and processed as mentioned in (A). Concentration of [^{3}H]DHE was 8 nM.

a K_d of ~4 nM and maximum binding of 35 fmol/mg protein. The binding of [^{3}H]DHE at 37°C and 23°C for various time intervals is shown in fig.1B. The binding is rapid reaching maximum in 20 min at 37°C and in 10 min at 23°C. The potency of various adrenergic ligands to displace [^{3}H]DHE binding in membranes was retained in the solubilized fraction. Fig.2 shows the displacement of [^{3}H]DHE binding from the receptor by various adrenergic agonists and antagonists. Half-maximal inhibition of [^{3}H]DHE binding was exhibited at 1×10^{-6} M, 8×10^{-7} M and 2×10^{-4} M for epinephrine, norepinephrine and isoproterenol, respectively. Among the antagonists yohimbine was potent in displacing the bound [^{3}H]DHE. Prazosin (α_1-antagonist) was without any effect on the displacement of [^{3}H]DHE from the binding sites. In addition, the membrane receptors bound [^{3}H]yohimbine which can be displaced >90% by non-radioactive yohimbine and 65–70% by phentolamine and epinephrine (not shown). This confirms [14] the observation made in the adrenocortical carcinoma membranes that these α-adrenergic receptors belong to α_2-subclassification. Propranolol (β-antagonist) did not have any effect on [^{3}H]DHE binding.

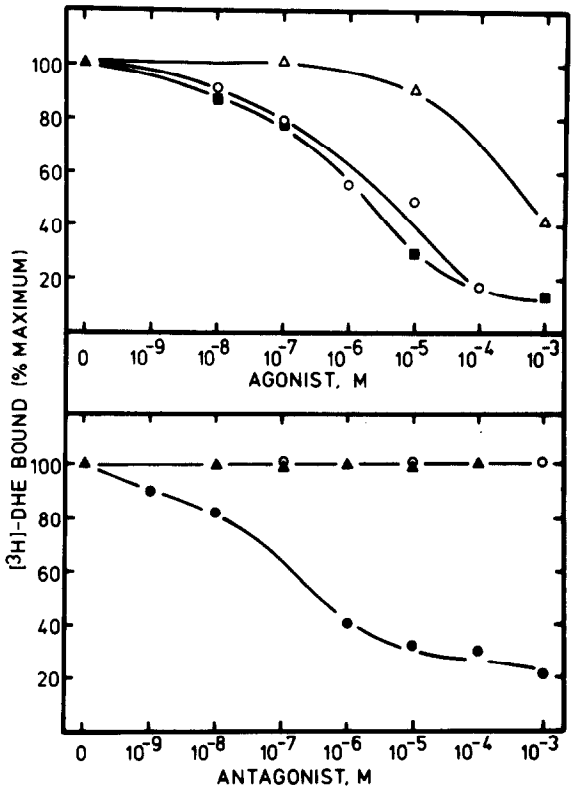

Fig.2. Displacement of bound [^{3}H]DHE from solubilized receptors by various agonists (top) and antagonists (bottom). Specific binding of [^{3}H]DHE was measured in the presence and absence of different concentrations of various agonists and antagonists. Assay conditions are the same as mentioned in fig.1A. Concentration of [^{3}H]DHE was 8 nM. Top: (○) epinephrine, (■) norepinephrine, (△) isoproterenol. Bottom: (●) yohimbine, (▲) prazosin–HCl, (○) propranolol–HCl.

4. Discussion

The native adrenocortical carcinoma cell membranes contain ACTH-, α-adrenergic and β-adrenergic receptors [5,6,15]. The treatment of the crude membranes with CHAPS selectively solubilizes receptors having characteristics of true α-adrenergic receptors. These bind [^{3}H]DHE rapidly with a high affinity (K_d 4×10^{-9} M), in a saturable fashion (35 fmol/mg protein) and the potency of α-agonists and antagonists to displace [^{3}H]DHE is higher than that of β-adrenergic agonists and antagonists.

That the α-adrenergic binding activity is indeed soluble was established, since it neither sediments at 105 000 × g for 1 h nor does it show any membrane characteristics upon electron microscopic examina-

tion (not shown). Since the presence of ACTH- or β-adrenergic receptors was not detected in the solubilized preparation, these studies demonstrate that the adrenocortical carcinoma α-adrenergic receptors are not only solubilized by the CHAPS treatment, but they also retain the original α-adrenergic characteristics of the native membranes.

Based on the binding studies of receptors with specific radioactive ligands, in many tissues the α-adrenergic receptors have been classified into α_1 and α_2 subtypes [16–18]. α_2-Adrenergic receptors show higher affinity for yohimbine and little affinity for α_1-antagonist prazosin and WB-4101. However, the reverse is the case for α_1-adrenergic receptors. Based on these criteria the solubilized adrenocortical carcinoma membranes are exclusively of α_2-subtype, since they have a high affinity for yohimbine (EC_{50} 10^{-7} M) and no affinity for prazosin. It is generally believed that α_2-receptors predominantly reside on presynaptic sites on the nerve terminals and their function is the inhibition of norepinephrine release by feedback mechanism [17,19]. However, these receptors coexist with α_1-receptors in rat heart membranes [20], rat brain [21] and hamster adipocytes [22], and predominantly [23] or exclusively in human platelets [24–27].

During the course of this investigation, solubilization of the human platelet α_2-adrenergic receptors by digitonin treatment was reported [25,27]. There is, however, one important difference between the characteristics of the solubilized receptors of platelets and adrenocortical carcinoma membranes. The solubilized platelet receptors show a reduced affinity for agonists as compared to the native receptors [25,27]; in contrast, the α_2-agonist and antagonist affinity for the native and solubilized carcinoma membrane receptors remains unchanged. Such a difference between the native and solubilized platelet α_2-receptor has led to the interpretation that the digitonin treatment results in the loss of a receptor component protein that is essential for the interaction of the α_2-receptor with the GTP-binding protein [25,27]. Such an interpretation is obviously not necessary in the case of the solubilized adrenocortical carcinoma membrane receptors prepared by the CHAPS treatment.

The molecular mechanism by which α_2-adrenergic receptors exhibit their biological activity is not known. Since these receptors in many tissues examined inhibit adenylate cyclase [10,11], it has been proposed that α_2-receptors are coupled to the adenylate cyclase in an inhibitory manner. In such a hypothesis, both the β-adrenergic and α_2-adrenergic agonists will exhibit their biological activity by interacting with the adenylate cyclase, the β-adrenergic agonist stimulating it and the α_2-adrenergic agonist inhibiting it.

The results with intact isolated adrenocortical carcinoma cells indicate that the epinephrine-activated rise of cyclic GMP is mediated by the α-adrenergic receptors [8]. The demonstration that these α-receptors are exclusively of the α_2-subtype in native membranes indicates that the epinephrine-activated rise of cyclic GMP in intact cells is mediated by the α_2-adrenergic receptors. This then indicates that the α_2-adrenergic receptors are coupled to guanylate cyclase and the transmembrane signal induced by the α_2-agonist occurs by its interaction with the α_2-adrenergic receptors which in turn results in the rise of cyclic GMP.

All these results indicate that the α_2-adrenergic coupled guanylate cyclase system might be one of the means of transmitting the catecholamine-sensitive biological response in the adrenocortical carcinoma cell.

Acknowledgements

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