Regulation of Beta-Adrenergic Receptors and Calcium Channel Agonist Binding Sites in Cultured Human Embryonal Smooth Muscle Cells

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Abstract. Recent electrophysiological studies with cell membrane patches of cardiac myocytes and an electrically excitable cell line derived from rat pituitary tumor suggested that voltage activated calcium channels must be phosphorylated to respond to membrane depolarization (Armstrong and Eckert 1986; Trautwein and Kameyama 1986). In view of the "phosphorylation hypothesis" we investigated the adenylate-cyclase activity, the characteristics of beta-adrenergic and calcium channel agonist binding sites in control and desensitized (exposure to isoproterenol) human embryonal cells (HEC), and in fragmented membrane preparations of canine coronary smooth muscle. Our results suggest that down-regulation of the membrane-bound beta-adrenergic receptors, induced by isoproterenol in human embryonal cells and also in adult canine vascular tissue, results in physical translocation of beta-adrenergic binding sites into the light membrane fraction. This phenomenon is accompanied with an increased intracellular concentration of cAMP in and an increased binding of the calcium channel agonist (^{3}H) BAYK 8644 to both HEC and canine smooth muscle membrane preparations. It could be concluded that phosphorylation of beta-adrenergic receptors regulates not only the beta subcellular distribution of the beta receptors but also the availability of calcium channel agonist binding sites in the cellular membrane.

Key words: Calcium channel — Beta-adrenergic receptors — Human embryonal cells — Coronary artery smooth muscle — (^{3}H) BAY K 8644-(^{3}H) DHA-(^{3}H) CGP 12177 binding

Introduction

Considerable experimental evidence has accumulated during the last few years to indicate that the activity of membrane potential-dependent Ca^{2+} channels is

effectively controlled by signals from receptors on the cell surface. Physiological studies indicate that voltage-sensitive Ca^{2+} channels are regulated by cAMP and protein phosphorylation (Armstrong and Eckert 1986; Schmid et al. 1985; Reuter 1983). Moreover, beta-adrenergically induced accumulation of cAMP in the myocardial cell has been reported to be associated with the increased probability of Ca^{2+} channel openings (Kameyama et al. 1985). There is now general agreement on the presence of beta-adrenergic receptors also in the coronary smooth muscle (Vatner et al. 1986; Dřímal 1988). Stimulation of beta-adrenergic receptors in excitable tissue is accompanied with accumulation of cAMP in the cells and in vascular smooth muscle, with phosphorylation of calmodulin-dependent myosin light chain kinase. The phosphorylated kinase has a lower affinity for calmodulin which results in marked vascular relaxation (Benovic et al. 1985). The activation step appears to involve binding of the agonist to beta-adrenergic receptor and reversible coupling of activated beta-adrenergic receptor with adenylate cyclase. These processes have been shown to be intimately associated with sequestration (downregulation) of beta-adrenergic receptors from the cell surface via a cAMP independent process. The phosphorylated state of beta-adrenergic receptor regulates its functional coupling to adenylate-cyclase, subcellular translocation and final recycling of the receptor to the cell surface during the process of agonist-induced desensitization (Hathaway et al. 1981).

To understand better this regulation mechanism and its relation to beta-adrenergic receptors and calcium channels in the vascular smooth muscle, we studied the density of beta-adrenergic receptors, cAMP accumulation and Ca^{2+} channel agonist binding sites in control and desensitized human embryonal cells (HEC), and in the canine coronary smooth muscle.

Materials and Methods

Chemicals: (5-methyl)-(^{3}H)BAYK 8644, specific activity 71.7 Ci/mmol (NEN Research Product, DuPont Boston, Ma.USA), 1(propyl-2, 3-T) (^{3}H)dihydroalprenolol, specific activity 60 Ci/mmol (IZINTA, Hungary), nitrendipine (Bayer AG, FRG), nitrendipine (Research Inst. Drugs, Modra, Czechoslovakia), (−)-(^{3}H)-CGP-12177, specific activity 34 Ci/mmol (Amersham, England), N-etyl-maleimide (Fluka, Switzerland), propranolol (ICI, England). All other materials were from sources as reported elsewhere (Dřímal et al. 1988).

Membrane fraction of the coronary smooth muscle. Young dogs (5—9 kg) were anesthetized with sodium pentobarbital (30 mg . kg^{-1} intravenously), arteficially respirated, the heart was quickly removed. The circumflex coronary artery was cannulated with a Philliphorm ureteral catheter and dissected from the ventricular wall. The artery was cut into 1 × 1 mm pieces and homogenized in nine volumes of ice cold 0.35 mol . l^{-1} sucrose and 5 mmol . l^{-1} Tris-HCl (pH = 7.51). The preparations were treated with eight 10 s strokes in an Ultraturrax Homogenizer. The homogenates were

pressed under vacuum through stainless steel wire mesh and centrifuged at 1500 × *g* for 10 minutes. The supernatant was further centrifuged (at 4 °C) in MOM Ultracentrifuge (Hungary) at 105.000 × *g* for 40 minutes. The resulting pellets were resuspended in assay buffer (50 mmol . l^{-1} Tris-HCl, pH = 7.51).

Cell culture. Human embryonal fibroblasts (HEC) were generously donated by Dr. J. Zavada (Institute of Virology, Slovak Academy of Sciences, Bratislava). The cell type used was characterized elsewhere (Zavada et al. 1986). Serum for cell cultures and other cell culture reagents were from Gibco (Grand Island, N. Y., USA). Catecholamines were from Fluka. HEC were cultured at 37 °C in an atmosphere of 10 % CO_2 in complete media consisting of Dubleco's modification of Eagle's medium with glucose, supplemented with 10 % fetal bovine serum. Cell monolayers were washed twice with cultivation medium and then incubated shortly with serum free media containing tritiated ligands and competing compounds.

Binding assays. (^{3}H)DHA, (^{3}H)CGP 12177 and (^{3}H)BAYK 8644 binding to membranes and to the microsomal fraction of the coronary smooth muscle was measured as described previously (Dřimal et al. 1988). The specific binding, which represented 85.0; 72.5; and 71.0 % of total binding, respectively, was determined by subtracting binding in the presence of 10^{-5} mol . l^{-1} alprenolol (in experiments with (^{3}H)DHA and (^{3}H)CGP 12177) or in the presence of 1.0 μmol . l^{-1} nitrendipine (in experiments with (^{3}H)BAYK 8644) from our values measured in the absence of the antagonists.

Radioligand binding to intact cells. At the end of a 16 h incubation period both control and desensitized cells (10^{-5} mol . l^{-1} isoproterenol added to the incubation solution) were scraped of the plates and harvested onto Whatman GR/C glass fiber filters. The cells were washed twice with 4.5 ml of assay buffer and the radioactivity bound on filters was collected in 10 ml of scintillation cocktail (LSD, Spolana Neratovice, Czechoslovakia) in a Packard 3-Carb Model 300 (Downes Grove, Ill., USA) at an efficiency of 40 %. For all assays involving small amounts of proteins one by one probe manifold filtration was carried out as described elsewhere (Dřimal 1988).

Adenylate-cyclase assays. Adenylate-cyclase assays were performed as described by Dřimal et al. (1987), except that cell monolayers were washed with lysis buffer, the cells were then homogenized, the homogenate was centrifuged and the cyclic-AMP content in the membranes was determined by the (^{125}I)-method (IRAPRA, Prague, Czechoslovakia). Protein was assayed by the method of Lowry (1951).

Results

Both high affinity ($K_D = 0.55 \pm 0.4$ nmol . l^{-1} and low capacity ($B_{max} = 112 \pm 5.0$ fmol . l^{-1} protein), and low affinity (34.5 ± 7 nmol . l^{-1}) and high capacity ($B_{max} = 1470 \pm 67$ fmol . mg^{-1} protein) binding sites could be demonstrated with (^{3}H)-DHA in intact HEC. The binding was reversible and stereospecific as confirmed by displacement experiments with (−) isomers of the antagonists nad (+) propranolol. Fig. 1. shows the Scatchard transformation of saturation binding experiments on HEC.

In displacement experiments (−) propranolol and (−) exaprolol effectively displaced (^{3}H)DHA specifically bound to both the high and low affinity sites. The order of potency was (−)exaprolol ≥ (−) propranolol ≫ (+) propranolol ≥ (+) propranolol ≥ BL 343 AC. The K_i values for the (−) isomers of antagonists studied were consistent with the results of the former experiments

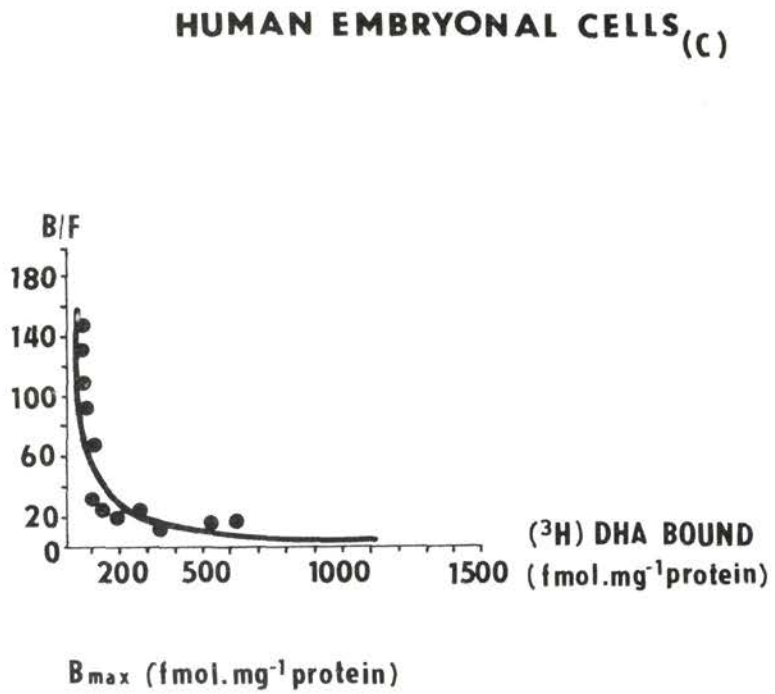

Fig. 1. Scatchard plot of specific (^{3}H)DHA binidng to human embryonal cells (HEC). The curvilinear plot suggests two clases of binding sites on young embryonal smooth muscle cells ($n = 6$). B_{max} and K_D values for the high affinity sites were 112 ± 5 fmol . mg^{-1} protein and 0.55 ± 0.4 nmol . l^{-1} respectively, and those for the low- affinity sites were 1470 ± 67 fmol . mg^{-1} protein and 34.5 ± 7 nmol . l^{-1}. Experiments were performed in triplicate. Values are mean $\pm$ S.E.M.

Table 1. Inhibition of (^{3}H)DHA binding in human embryonal cells. Inhibition experiments were performed by incubating control cells in the presence of increasing concentrations of unlabeled antagonists. K_i values were calculated by the formula: $K_i = IC_{50}/(1 + F/K_d)$.

Antagonist	K_i (nmol . l^{-1})	n_H
(−) propranolol ($n = 6$)	1.2 ± 0.2	0.98
(−) exaprolol ($n = 5$)	1.4 ± 0.3	0.94
(+) propranolol ($n = 3$)	170.0 ± 22	0.67
BL 343 AC	4828.0 ± 126	0.56

n_H = Hill coefficient, values are mean $\pm$ S.E.M., n = number of experiments.

Table 2. Cyclic AMP content of human embryonal cell membranes after exposure to isoproterenol. cAMP content of human embryonal cells in culture was assayed after 16 h incubation with 1.0 μmol . l^{-1} and after final lysis.

cAMP	Control	Desensitized
pmol/mg protein	4.6 (n = 10) ± 1.1	5.8 (n = 12) ± 1.3

Values are mean ± S.E.M.

Table 3. Characteristics of binding of (^{3}H)CGP 12177 to membrane and microsomal fractions of canine coronary artery. B = % of control B_{max}; and K_D (nmol . l^{-1}). Control coronary artery preparation (Control) and pretreated (isoproterenol 1.0 μg . min^{-1}, intracoronary) coronary artery (ISO).

	Control		ISO	
	B(%)	K_D	B(%)	K_D
Membrane fraction (n = 6)	81.9 ± 11.0	0.3 ± 0.05	42.6° ± 10.0	11.2° ± 4.0
Microsomal fraction (n = 6)	17.2 ± 0.9	0.8 ± 0.5	57.6° ± 9.0	10.5° ± 1.7

n = number of experiments, each experiment in triplicate measurements. Values are: Mean ± S.E.M. ° — significant difference from control $p < 0.05$.

(Table 1). Cultured HEC exposed to beta-adrenergic agonist isoproterenol showed a decrease in the cell surface receptors. Saturation binding of (^{3}H)DNA showed that in desensitized cells beta-adrenergic receptors have been down-regulated to 1/3 of their initial value; B_{max} of the desensitized cells was 510 ± 25 fmol . mg^{-1} protein, and the affinty constant suggested the presence of only low affinity binding sites (K_D = 22.4 ± 7.0 nmol . l^{-1}).

To analyze the process of down-regulation of beta-adrenergic receptors in the human embryonal cells in more detail, the results of (^{3}H)DHA binding to intact living cells were compared with those of (^{3}H)CGP 12177, a hydrophilic beta-antagonist that does not penetrate into cells and is believed to label selectivelly only surface beta-andrenergic receptors (Maisel et al. 1985). The hydrophilic beta-antagonistic radioligand labeled significantly fewer receptors in desensitized human embryonal cells with B_{max} values of 989 ± 19 fmol . mg^{-1} protein for control and 245 ± 67 fmol . mg^{-1} protein for desensitized cells ($P < 0.05$).

To confirm the above results, the whole cell preparations were exposed to

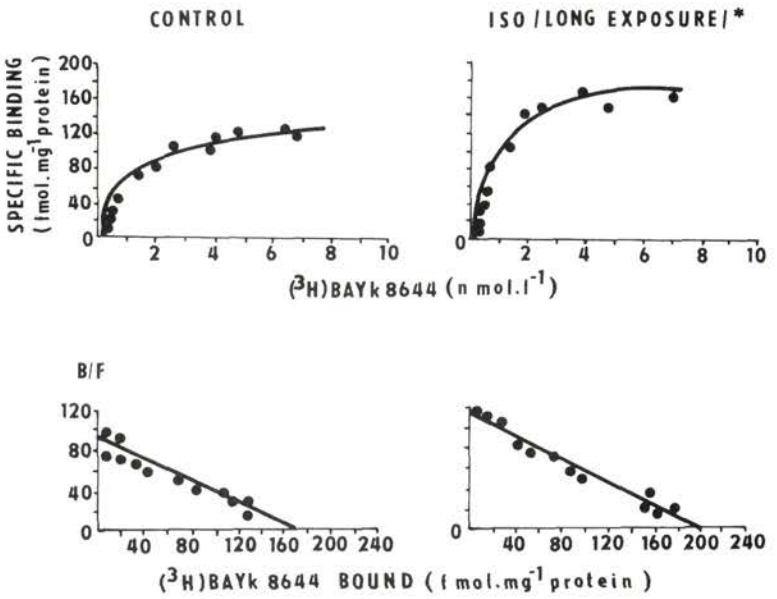

Fig. 2. Specific binding of (^{3}H)BAYK 8644 to intact (control) and desensitized (ISO) cells exposed to isoproterenol (1.0 μmol . l^{-1} for 16 h). The control and desensitized human embryonal cells were incubated with increasing concentrations of (^{3}H)BAYK 8644 in the presence and in the absence of cold nitrendipine (1.0 μmol . l^{-1}), (*Top*); *Bottom*: Scatchard plots of specific binding. For explanation and symbols see text.

isobutylmethylxantine, lysed and plasma membrane and microsomal fractions were prepared from both control and isoproterenol treated cells. For this purpose the cell preparations were separately centrifuged on 15/50 % sucrose density gradient. The fractions obtained were then assayed for the presence of adenylate-cyclas. The basal cAMP content in the membrane fraction of human embryonal cells was 1.5 ± 0.3 pmol . mg^{-1} protein. Incubation with isoproterenol produced an immediate increase in the cyclic AMP content in that fraction. Maximal increase in the cyclic AMP content occurred with 60 s exposure (8.7 ± 1.2 pmol/mg protein, $P < 0.05$). A slight but significant drop of the cAMP content was obseved after 1.5 — 20.0 min incubation. After exposure of HEC to isoproterenol (16 h was the longest interval studied) the stimulated adenylate-cyclase activity remained significantly above the basal levels (Table 2).

The results obtained with radioligand binding to intact living cells were compared with the data for fragmented canine coronary smooth muscle and with the distribution of beta-adrenergic receptors in different subcellular fractions. For this purpose we used classical fragmented preparations of coronary smooth muscle and the hydrophilic beta-adrenergic antagonist (^{3}H)CGP 12177.

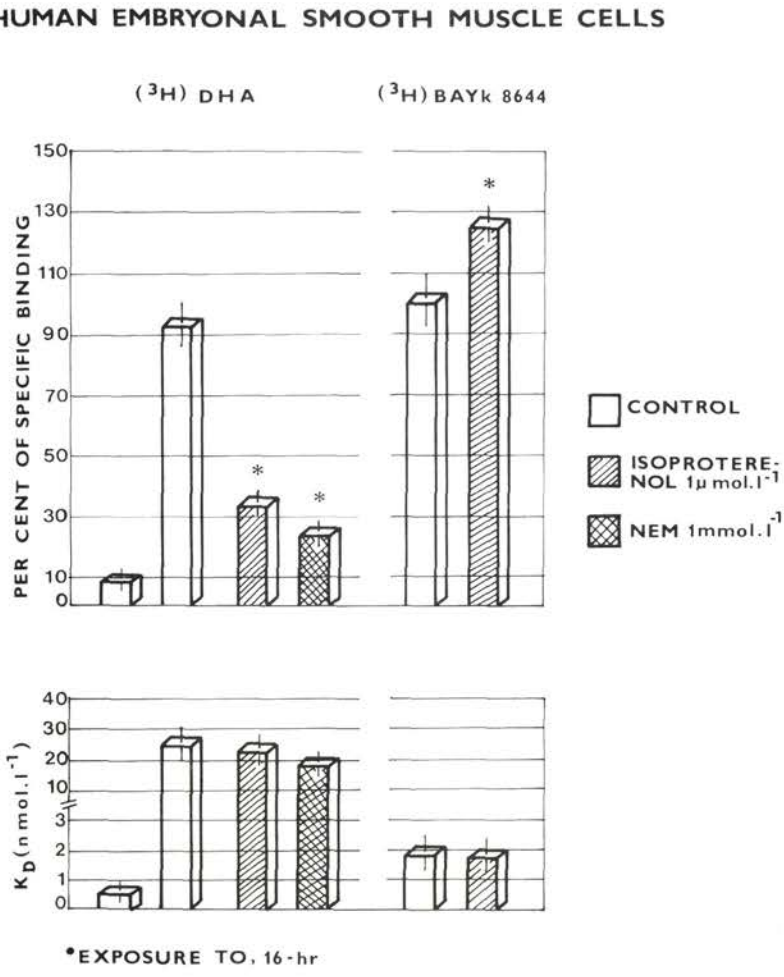

Fig. 3. Summary of the results obtained with specific binding of (^{3}H)DHA and (^{3}H) BAYK 8644 in control (□) and desensitized (▨) human embryonal cell lines. NEM (N-ethyl-maleimide) (⊠): whole cell preparations exposed to N-ethylmaleimide (1.0 mmol . l^{-1}) prior to desensitization.

The results of this latter test series confirmed those obtained on whole cell preparations: when the coronary artery was exposed to isoprenaline by intracoronary infusion, 1.0 μg . kg^{-1} . min^{-1}) and subsequently fragmented and fractioned, a large portion of plasma membrane receptors disappeared from that fraction (Table 3) and appeared in the microsomal fraction. In contrast, the plasma membrane control coronary artery fractions showed only negligible densities of beta-adrenergic receptors in the microsomal membranes.

As a next step in studying beta-adrenergic control of calcium channel agonist binding sites in human embryonal smooth muscle we analyzed (^{3}H)BAYK 8644 calcium channel agonist binding sites for both control and desensitized (isoprenaline exposed) human embryonal cells. (^{3}H)BAYK 8644 binding to human embryonal cells was found to be specific and saturable. The maximal number of binding sites (B_{max}) for control whole cell preparations was 172 ± 8.1 fmol . mg^{-1} protein and the binding affinity constant was 2.1 ± 0.2 nmol . l^{-1}. For isoproterenol-exposed human embryonal cells B_{max} was 198 ± 6 and affinity constant 2.0 ± 0.1 nmol . l^{-1} (Fig. 2).

In competition experiments nitrendipine displaced (^{3}H)BAYK 8644 from the high affinity binding sites with a K_i values 0.3 nmol . l^{-1}. The apparent Hill slope (n_H) was 0.96, suggesting a common high affinity site of action for the Ca^{2+} channel agonist and nitrendipine in the whole cell preparation.

Discussion

In this report we give the basic characterization of beta-adrenergic and calcium channel agonist binding sites of human embryonal vascular cells and fragmented preparations of canine coronary artery exposed to isoprenaline (short-term exposure as well as chronic exposure; maximally 16 hr). The beta-adrenergic and calcium channel agonist binding sites were identified in the present study with two beta-adrenergic antagonists, (^{3}H)DHA and (^{3})CGP 12177, and with an agonist a 1,4-dihydropyridine calcium channel radioligand (^{3}H)BAYK 8644.The results provide direct evidence for the existence of a significant pool of binding sites with characteristics of beta-adrenergic receptor and calcium channel agonist binding sites in the whole cell prepration of HEC. The population of specific (^{3}H)DHA and (^{3}H)CGP 12177 binding sites in the canine coronary smooth muscle membranes appears to be saturable and to have a low density.

The total number of binding sites with characteristics of beta-adrenergic receptors, identified in the canine coronary artery, principally agree with the data reported by Vatner et al. (1986) for bovine and by Schwartz and Velly (1983) for porcine coronary artery.

One remarkable finding of the present study concerns the positive correlation between the decreased density of beta-adrenergic receptors and the increase in number of calcium channel agonist binding sites in both the human embryonal cells and the membrane preparations of the coronary artery after isoproterenol stimulation. This beta-adrenoceptor activation and regulation in human embryonal cells has not been reported as yet, although similar correlation, that is beta-adrenergic stimulation and 1,4-dihydropyridine sensitive

$^{45}Ca^{2+}$ uptake, has been reported (Schmid et al. 1985). Trautwein and Kameyama (1986) in electrophysiological experiments suggested that phosphorylation of protein is necessary to increase the probability of the "available" state of Ca^{2+} channels on beta-adrenergic stimulation. In agreement with these observations our study indicate that exposure of human embryonal cells, or exposure of adult canine coronary smooth muscle, to isoprenaline results in a desensitization response that is almost indistinguishable from the desensitization observed by other authors in different types of living cells (Strader et al. 1984; Hoyer et al. 1984; Clark 1986).

The curvilinear Scatchard plot of (^{3}H)DHA binding obtanined in the present study with HEC may reflect the complexity of physiochemical properties of (^{3}H)DHA binding in this preparation (Alexander et al. 1982). The relevance of this phenomenon to the various stages of regulation of beta-adrenergic receptors in human tissue remains unknown. Another interesting problem concerns the stoichiometry of the down-regulated beta-receptors and the density of calcium channel agonist binding sites. The summarized results of our exprimments show beta-adrenergic down regulation to 32 % of the initial values in the whole cells and an only 15 % increase in the calcium channel binding sites. Our studies also show that out of the beta-adrenergic receptors labeled by and available to (^{3}H)DHA, only 62.5 % in control and 15.5 % in desensitized whole cell preparations are available to the hydrophilic beta-adrenergic ligand (^{3}H)CGP 12177.

Similarly to the results of Stadel et al. (1983) also in this study long time treatment of whole cell preparation with isoprenaline resulted in an apparent movement of beta-adrenergic receptors from the plasma membrane to a light vesicle fraction.

In conclusion, our experiments show that beta-adrenergic receptor down-regulation in human embryonal cells and in canine coronary smooth muscole membranes, induced by beta-adrenergic agonist isoproterenol, is accompanied with an increased calcium agonist binding; this may support the idea of a common mechanism of desensitization response-regulation of calcium channels and thus the validity of the "phosphorylation hypothesis" also in vascular tissues.

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Final version accepted February 20, 1989