

## ULTRASTRUCTURAL PROBING OF $\beta$ -ADRENORECEPTORS ON CELL SURFACES

Daphne ATLAS, David YAFFE<sup>†</sup> and Ehud SKUTELSKY\*

*Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, <sup>†</sup>Department of Cell Biology and \*Department of Ultrastructure, The Weizmann Institute of Science, Rehovot, Israel*

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### 1. Introduction

Establishment of several myogenic cell lines from rat skeletal muscle was achieved by serial passages in vitro of myoblasts from day 2 rat thigh muscles [1–3]. Rat skeletal muscle cell lines L6D [5], H6 [5] and L84 [6] were shown to be highly responsive to stimulation by (–)adrenaline (NA) to produce high levels of cyclic AMP (cAMP). Cyclic AMP is an important participant in the life cycle of the cell and its level is a direct function of the various stages of growth. A significant decrease in the level of cAMP was observed [5] after fusion to myotubules. The large increase in intracellular cAMP levels subsequent to the noradrenergic stimulation of L6 cells [4] indicates the presence of a large population of  $\beta$ -adrenergic receptor sites on the cell surface. Indeed, direct binding studies with <sup>125</sup>I-labeled  $\pm$ hydroxybenzylpindolol ([<sup>125</sup>I] $\pm$ HYP) confirmed the presence of a large number of  $\beta$ -adrenergic receptor sites on L6P cells [7]. Therefore, the L6P cells were used to identify the  $\beta$ -adrenergic receptor subunit with a tritiated irreversible  $\beta$ -blocker [8]. An attempt to study the distribution of the  $\beta$ -adrenoreceptors on the cell surface was carried out by the use of the fluorescent  $\beta$ -blocker, 9-amino-acridino-propranolol (9-AAP), on L6P cells [9]. Although a non-random distribution of  $\beta$ -adrenoreceptor sites was visualized by fluorescence microscopy using 9-AAP, no information could be obtained as to their ultrastructural distribution.

Autoradiographic studies using <sup>125</sup>I-labeled  $\alpha$ -bungarotoxin on embryonic chick skeletal muscle, grown in culture, show a homogeneous distribution of  $\alpha$ -bungarotoxin binding sites in newly fused myotubules but during subsequent differentiation,

clusters of acetylcholine receptors appear [10]. We report here on a technique to study the ultrastructural distribution of the  $\beta$ -adrenoreceptor sites on cell surfaces, using a newly synthesized potent  $\beta$ -blocker, a biotin-propranolol analog (BPA). After the application of this ligand to cell surfaces, the preparation is exposed to an avidin–ferritin conjugate, allowing the ultrastructural visualization of  $\beta$ -adrenoreceptor sites. The use of the avidin–ferritin conjugate as an ultrastructural probe to study membrane constituents has been successfully applied [11–14]. The improved technique [15,16] for the conjugation of avidin to ferritin, forming single-paired active ferritin–avidin conjugates by reductive alkylation, has recently been developed and was applied in this study. The main features of the method presented here is the use of a new  $\beta$ -blocker (fig.1) with a biotin moiety. This potent  $\beta$ -antagonist (fig.2) is applied to the cells that are then exposed to ferritin–avidin before or after fixation with Karnovsky fixative [12], and then processed for visualization in thin sections in the electron microscope [15,16].

### 2. Materials and methods

#### 2.1. Biotin-propranolol analog

This was prepared from biotin-*N*-hydroxysuccinimide ester and NHNP-E, prepared as in [21] (see fig.1 legend).

#### 2.2. The $\beta$ -adrenergic characteristics of the new $\beta$ -blocker

The new  $\beta$ -antagonist (D. A., in preparation) is an analog of propranolol, ( $\pm$ ) *N*-[(-)-hydroxy-3(-1-naph-

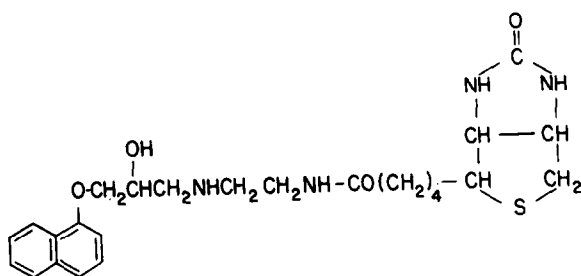


Fig.1. The structure of biotin-propranolol analog (BPA). *N*-[2-hydroxy-3-(1-naphthyloxy)-propyl] ethylenediamine (NHNP-E) [21] was coupled with the *N*-hydroxysuccinimide ester of biotin (D. A. in preparation).

thyloxy)-propyl]-*N'*-biotinyl-ethylenediamine (fig.1). It is a potent  $\beta$ -blocker, as determined by its capacity to inhibit competitively the (-)adrenaline-stimulated adenylate cyclase activity. The  $K_d$  was calculated from the concentration of this compound required to inhibit 50% of the (-)adrenaline-stimulated activity in turkey erythrocyte membranes (fig.2), and was  $(1.8 \pm 0.8) \times 10^{-8}$  M (fig.2) for the BPA.

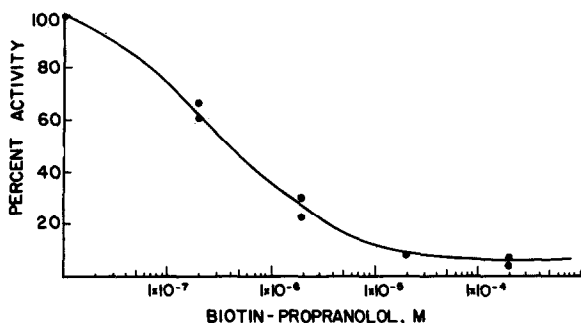


Fig.2. Inhibition of (-)adrenaline-stimulated activity of adenylate cyclase by BPA. The dissociation constant ( $K_d$ ) was calculated as presented in the formula [12,13]:

$$K_d = \frac{S_{50}}{L} \times K_1$$

where  $S_{50}$  is the concentration of the BPA needed to inhibit 50% of the total effect of (-)adrenaline;  $K_1$  is the dissociation constant of (-)adrenaline; and  $L$  is the concentration of (-)adrenaline in the adenylate cyclase assay.  $S_{50} = 3.6 \times 10^{-7}$  M;  $K_d = (1.8 \pm 0.8) \times 10^{-8}$  M.

### 2.3. Procedure for labeling $\beta$ -adrenergic receptor on cell surfaces

L84 rat skeletal myogenic muscle cells, grown in culture, were cultivated in nutritional medium supplemented with 10% serum [2]. In this serum the L84 continued to proliferate to very high densities without fusion to multinucleated fibers [2]. The medium was discarded and the cells were washed 2 times with saline. Then the BPA was applied to the petri dish at  $5 \times 10^{-5}$  M, in a layer that covered the cells, and the cells were incubated at room temperature. After 5 min the cells were washed 2 times with saline and exposed to a solution of ferritin-avidin conjugate, then centrifuged ( $10\,000 \times g$  30 min) to remove any possible aggregates. After 20 min at room temperature, the cells were thoroughly washed 6 times with saline and fixed with Karnovsky fixative [17]. The same results were obtained if the fixation of the cells was carried out immediately after the binding of the BPA to the cells and only then exposed to ferritin-avidin. Preincubation of the cells with ( $\pm$ )propranolol ( $1 \times 10^{-4}$  M) at room temperature for 10 min followed by washing, and the above procedure, served as a control to establish the specificity of BPA.

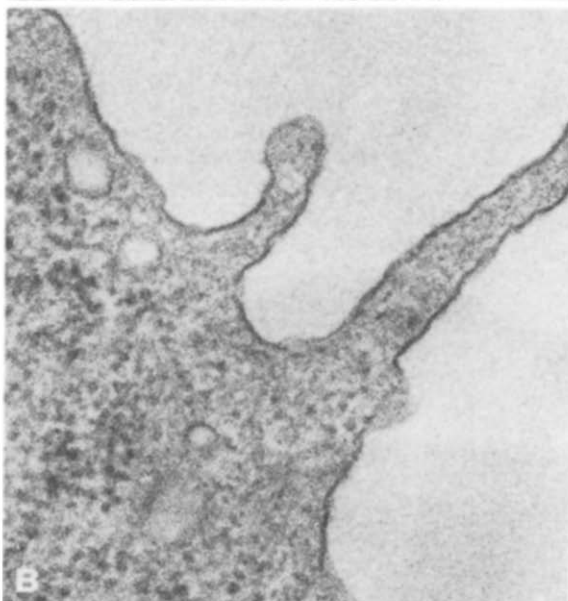
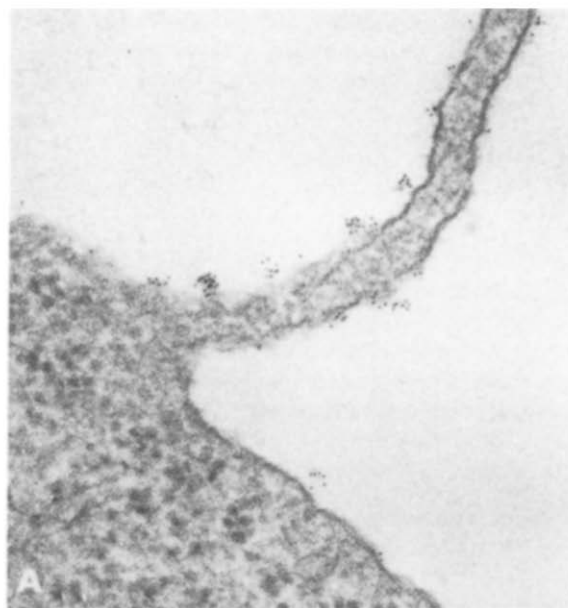
### 3. Results and discussion

Ferritin particles were dispersed on cells treated with BPA and ferritin-avidin conjugates (fig.3A) but almost no labeling was observed on cells pretreated with ( $\pm$ )propranolol (fig.3B). The frequency of attached ferritin particles was usually higher on cell microvilli as compared with the remaining cytoplasmic membranes (fig.3A). On tangential cell membranes there was a clear tendency of the ferritin particles to appear in clusters consisting of a variable number of particles. The average density of ferritin particles was  $71 \pm 19$  SD/ $\mu\text{m}^2$  (fig.3A). Since in other experiments [11-16] utilizing the technique of ferritin-avidin conjugates there was very little, if any, cluster formation, the cluster appearance on the L84 cells' surfaces may be relevant to the arrangement of the  $\beta$ -adrenergic receptors on the cells.

From fluorescence microscopy in *in vivo* studies using the fluorescent  $\beta$ -blocker [18-20], the fluorescence pattern observed on cell surfaces displayed an array of fluorescent beads, suggesting that the  $\beta$ -adrenergic receptors are clustered in domains. The present

results strongly suggest that the labeling by BPA is a specific one, as no ferritin was detected if prior treatment with propranolol was performed, and that the  $\beta$ -receptors tend to form clusters on the cell surface.

We would like to suggest that the use of biotinylated ligands can be applied as a general technique to study the distribution of hormone- and neurotransmitter-receptors on the cell surface.



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Fig. 3A,B. Distribution of attached ferritin–avidin conjugate on thin sections of cultivated L84 cells treated with biotinylated propranolol ( $5 \times 10^{-5}$  M) in the absence (3A) and in the presence of excess ( $\pm$ ) propranolol ( $1 \times 10^{-4}$  M (3B)). Note the relatively high ferritin density on membranes surrounding cytoplasmic protrusions (3A) and the absence of ferritin particles on the control cells (3B). The BPA and the propranolol treated cells were fixed for 1 h at 25°C with Karnovsky fixative, washed 6 times with saline, incubated for 30 min at 25°C with ferritin–avidin conjugate (1 mg/ml ferritin), washed 2 times with saline, and fixed with Karnovsky fixative. The cells were scraped from the petri dish using a razor blade, concentrated by centrifugation ( $10\,000 \times g$ ) into a pellet that was post fixed with 1%  $\text{OsO}_4$ , dehydrated and embedded in Epon 812. Attached ferritin particles were counted on electron micrographs of thin sections of perpendicularly sectioned membranes at a  $60\,000 \times$  magnification. The length of the perpendicularly sectioned membrane on which the number of the ferritin particles were counted, was measured on the micrographs with a map measurer. Magnifications: 3A, 3B,  $60\,000 \times$ .

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