FLUORESCENT VISUALIZATION OF β-ADRENERGIC RECEPTORS ON CELL SURFACES

Daphne ATLAS and Alexander LEVITZKI
Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel

Received 12 September 1977
Revised version received 31 October 1977

1. Introduction

Numerous cell types were shown to possess β-adrenergic receptors coupled to adenylate cyclase. Using radiolabeled high affinity β-adrenergic blockers, it became possible to determine the exact number of β-receptors per cell. Most cells [1–11] were found to possess a small number of receptors which varies between a few hundred to a few thousand receptors per cell. Recently it was demonstrated that rat skeletal myoblasts grown in culture (L6P cells) [12] and a strain of HeLa cells [13] possess a large number of β-adrenergic receptors. In this communication we report on the visualization of β-adrenoreceptor sites by the use of the fluorescent β-adrenergic blocker 9-aminoacridino-propanolol (9-AAP) [14,15]. It will be shown that the capability to visualize the β-receptors, using a fluorescent β-blocker, is limited by receptor density on the cell surface.

The fluorescent β-blockers 9-AAP and DAPN [14] have been used successfully to map β-adrenergic receptors in vivo [15–22]. In these studies the fluorescent β-blockers were first injected in vivo into the tail vein of rats or mice; then the organs were frozen, cut in a cryostat, and subsequently the sections were subjected to fluorescence microscopy. Using this technique distinct cells within the tissues examined which possess β-adrenergic receptors on their surface were identified [15–22]. These findings indicate that the receptor density of these cells is high enough to enable their visualization. An attempt will be made to correlate the findings in vivo with those reported in this study on cells grown in culture.

Abbreviations: 9-AAP, DL-*N*-(2-hydroxy-3-naphthyloxy-propyl)-*N'*-9-acridino-isopropylenediamine or 9-amino-acridinopropranolol; DAPN, DL-*N*-(2-hydroxy-3-naphthyloxy-propyl)-*N'*-dansylethylenediamine or dansyl analogue of propranolol

2. Experimental

2.1. *Growth of cells*

2.1.1. L6P Cells

Skeletal muscle cells (L6P cells) were grown in culture, as described [1]. Cells were removed from the growth bottles using EDTA and counted, as described [12]. Protein was determined in order to established no. cells/mg protein. Since trypsin is often used to remove cells from tissue culture dishes, it was also used to remove L6P cells from the tissue culture bottles in another set of experiments, according to the following procedure: The growth medium was removed from the tissue culture bottle and the cells were incubated with 50 ml 130 mM saline, containing 20 mM Tris–HCl buffer, pH 7.4, and 500 μg/ml trypsin for 30 min at 37°C. The cells were detached from the bottle, centrifuged at 500 × *g*, and washed twice with trypsin-free saline containing buffer.

2.1.2. HeLa Cells

Cells were grown for 3 days at 37°C in M-199 medium, containing 10% calf serum inactivated at 56°C for 30 min. Cells were removed from the growth bottle using a solution of 140 mM NaCl, 10 mM Tris–HCl, pH 7.4, 10 mM glucose, and 2 mM EDTA.

2.1.3. Turkey erythrocytes and S49 lymphoma cells

Turkey erythrocytes were prepared as described [12,23].

2.2. *Binding of [^{125}I]HYP*

[^{125}I]HYP binding to intact cells and to cell membranes was conducted as described [7,12].

2.3. *Protein determination*

Protein was determined using the method [24]. According to the protein determination, it was found that 1.0 mg total protein = 2.88 × 10^6 L6P cells and 7.1 × 10^5 HeLa cells, respectively.

2.4. *Fluorescence microscopy of intact cells*

Washed L6P cells or HeLa cells were suspended in 130 mM NaCl, 20 mM Tris—HCl, pH 7.4 and 10 mM glucose. Cells were mixed with 1.3 × 10^{-5} M 9-AAP with or without 3.3 × 10^{-6} M L-propranolol or D-propranolol. Fluorescence was viewed and photographs were taken using a Nikon fluorescence microscope equipped with a Nikon M-355 camera. The film, Kodak 400 ASA, was developed with the corresponding 400 ASA developer. Exposure time was 2 min using the B filters as fluorescence excitation filter. Pictures were taken within 10 min after mixing the cells with the fluorescent dye and the propranolol stereoisomers. The intensity of fluorescence was found to decay upon cell death, which may occur within 30 min subsequent to the exposure of the cells to fluorescent dye under the cover slide.

3. Results

3.1. *Binding of [^{125}I]HYP to intact cells and the effect of trypsin*

L6P cells and HeLa cells possess 80 000 receptors and 37 000 receptors, respectively (data not shown). In both cases the cells possess a single class of [^{125}I]-HYP receptor-binding sites.

Removal of L6P cells from the tissue culture bottle using trypsin results in a 5-fold reduction in the content of β-receptors as compared to cells removed by EDTA treatment (fig.1).

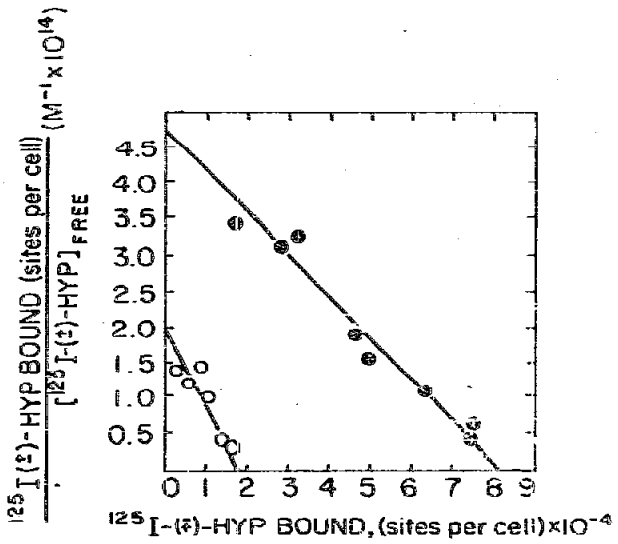

Fig.1. The binding of [^{125}I]HYP to intact L6P cells. The binding data is represented in the form of a Scatchard plot. The non-specific binding was measured in the presence of 1.0 × 10^{-5} M L-propranolol, as described [12]. Cells removed by trypsin treatment exhibited identical extent of non-specific [^{125}I]HYP binding as cells removed by EDTA. (●—●) L6P cells removed by EDTA treatment. (○—○) L6P cells removed by trypsin treatment.

3.2. *Stereospecific fluorescence visualization of β-receptors*

In fig.2 it can be seen that L6P and HeLa cells bind 9-AAP, presumably to the β-receptors. The appearance of intense fluorescence dots can be inhibited by L-propranolol but not by D-propranolol. The fluorescence pattern observed when the cells are mixed with 9-AAP in the presence of L-propranolol is rather faint. On the other hand, when the cells are mixed with 9-AAP in the presence of the inactive stereoisomer D-propanolol, the intense fluorescence pattern remains essentially unchanged. The hazy fluorescence observed in the presence or absence of L-propranolol is evenly distributed on the cell surface, and probably represents non-specific solubilization of 9-AAP in the cell membrane. Similar experiments using S49 glioma cells, which possess about 200—300 receptors/cell [8], and turkey erythrocyte ghosts, which possess about 1000 receptors/cell [1,12], did not reveal fluorescent dots except for the non-specific, evenly-distributed faint fluorescence.

4. Discussion

4.1. *Number of β-receptors per cell*

In this study we have shown that the fluorescent

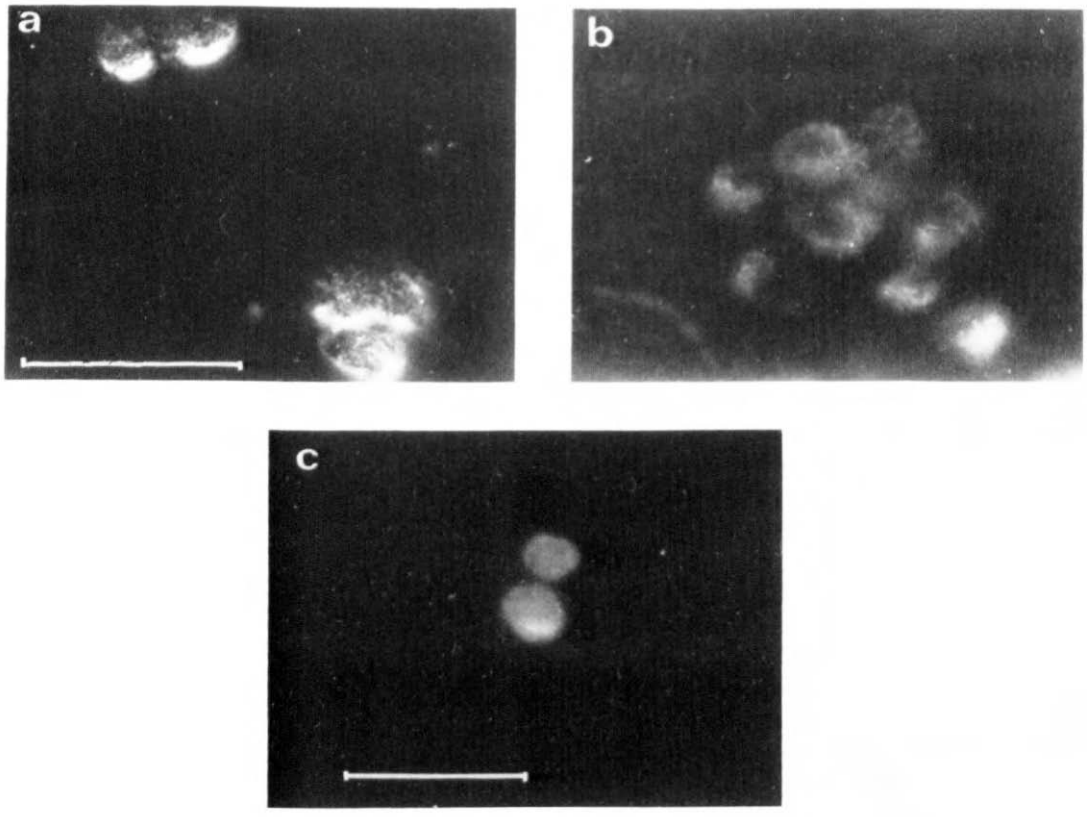

Fig.2. Fluorescence visualization of β-receptors on L6P cells and on HeLa cells. (a) L6P cells in the presence of 1.0×10^{-5} M 9-AAP. (b) L6P cells in the presence of 1.0×10^{-5} M 9-AAP and 3.0×10^{-6} M L-propranolol. (c) HeLa cells in the presence of 1.0×10^{-5} M 9-AAP. The bar in a–c represents 40 μm.

analogue of propranolol, 9-AAP, which binds stereospecifically to the β-adrenoreceptors, allows the visualization of the β-adrenergic receptors, provided that the density of the receptors is high enough. When cells or membranes possessing only a few receptors per μm^2 are treated with 9-AAP, no fluorescence can be detected. Thus, for example, turkey erythrocyte membranes, native turkey erythrocytes, or S49 lymphoma cells do not reveal specific fluorescence staining using 9-AAP (see also table 1). Skeletal myo-

Table 1
The density of β-adrenergic receptors in a number of cell types

Cell type	Receptors/cell	Average density (receptors/μm^2)
Turkey erythrocyte	1100[a]	8
S49 lymphoma	200–300[b]	<8
L6P (skeletal muscle)	80 000	160[c]
HeLa	37 000[d]	100–200[d]

[a] From [1,12]
[b] From [8]
[c] This study and [12]
[d] This study

blasts grown in culture (L6P cells) and HeLa cells possess 30–80-fold more receptors/cell (fig.1) than turkey erythrocytes (table 1). Considering the surface area of L6P cells and of HeLa cells used, it can be calculated that the receptor density is 12–20-fold higher than in turkey erythrocytes. All other types of cells, which were found to possess β-adrenergic receptors, were demonstrated to possess a receptor density equal or lower to that of turkey erythrocytes. It is clear, therefore, that the ability to visualize specific 9-AAP fluorescence depends directly on the density of the β-adrenergic receptors.

It is interesting that rat skeletal membranes prepared from the thigh muscle of two-day old rats possess only 0.38 pmol/mg β-receptors as measured by [^{125}I]HYP binding (D.A. and A.L., unpublished). The L6P skeletal muscle cells used in our studies are derived from this thigh muscle tissue [25].

4.2. *Stereospecificity of 9-AAP binding*

The fluorescent analogue is a competitive inhibitor for L-epinephrine in the adenylate cyclase reaction, and competitively inhibits [^{125}I]HYP binding to β-receptors [14]. In in vitro fluorescence experiments only L-propranolol and L-epinephrine are capable of displaying 9-AAP from the β-adrenergic receptor of turkey erythrocyte membranes [14]. The stereospecificity of 9-AAP binding is also revealed in the binding of the fluorescent compound to the intact L6P cells (fig.2). The active isomer L-propranolol displaces effectively the fluorescent β-blocker from the cell surface, leading to a significant reduction in fluorescence intensity, whereas D-propranolol is ineffective. The hazy fluorescence shown in the presence of L-propranolol probably reflects the non-specific solubility of the compound in the cell membrane. It should be pointed out that the faint fluorescence was a typical observation in all cells examined which possess low receptor density, such as S49 lymphoma cells and turkey erythrocytes.

4.3. *The number and distribution of β-receptors on cell surfaces*

The fluorescent analogues of propranolol, 9-AAP and DAPN, can be used successfully to localize β-adrenergic receptors in vivo [14–22]. In these experiments the fluorescent analogue was injected in vivo into the animal. A short time thereafter the animal was sacrificed, the organs frozen, and the cryostat sections visualized using fluorescence microscopy. In these studies it was demonstrated that only cell bodies possessing β-adrenergic receptors become stained with the fluorescent analogues. Cells such as turkey erythrocytes or S49 lymphoma cells, which possess only a few receptors per μm^2 (table 1), do not reveal any specific fluorescence when exposed to 9-AAP. Cells with high receptor density such as L6P cells or HeLa cells fluoresce strongly when exposed to 9-AAP. Therefore, cell bodies which fluoresce subsequent to injection of 9-AAP in vivo possess a high density of β-receptors. The fluorescence pattern observed in vivo indicates domains in which the receptors are clustered [19].

4.4. *Distribution of receptors*

From fig.2 it is apparent that the fluorescence dots distribution on the cell body is not homogeneous. The β-adrenoreceptors could be clustered in domains which are not evenly distributed on the cell membrane. Clustering could be induced by the fluorescent β-blocker but could pre-exist on the membrane. This question is currently under investigation.

4.5. *The turnover number of adenylate cyclase*

Skeletal muscle myoblasts grown in culture (L6 cells) exhibit a very high L-epinephrine dependent adenylate cyclase activity [26]. The value reported [27] was 500 pmol cAMP/mg cells/min. Assuming a stoichiometric relationship between the β-adrenergic receptor and the cyclase, a value of 2.88×10^6 cells (this study)/mg protein, and a turnover number for adenylate cyclase of 1400 min^{-1} [2], one can calculate that the L6 cells used [27] possess

$$\frac{500}{1400} \times \frac{6.03 \times 10^{23} \times 10^{-12}}{2.88 \times 10^6} = 75\,000 \text{ receptors/cell.}$$

The value found by direct [^{125}I]HYP-binding measurements to intact cells in this study, as well as [12], was found to be 80 000–84 000 receptors/cell. It appears therefore that the assumption made on the stoichiometric relationship between adenylate cyclase and the β-adrenergic receptor, originally made for the turkey erythrocyte cell [2], is valid and may represent a universal correlation between the receptor and the enzyme.

5. Conclusions

The density of β-adrenergic receptors on the surface of rat skeletal myoblasts grown in culture (L6P) and on HeLa cells was found to be almost two orders of magnitude higher than in all other cell types known to possess β-adrenergic receptors. The high density of β-adrenergic receptors on these cells allows their visualization by fluorescence microscopy using the high affinity fluorescent β-adrenergic blocker, 9-AAP. The fluorescence labeling of these receptors is stereospecific. From the fluorescence pattern observed, it is suggested that the β-receptors are clustered in domains. The β-adrenergic receptors of cells which possess a rather low density of receptors cannot be visualized by this technique. A comparison between these findings and the established ability of this compound to map β-adrenergic receptors in vivo was made.

Acknowledgements

This work was supported by a grant from the United States-Israel Binational Research Foundation (BSF), Jerusalem and partially by the Deutsche Forschungsgemeinschaft, FRG. The authors wish to thank Dr D. Yaffe and Miss R. Meller from The Weizmann Institute of Science, Rehovot, for growing the L6P cells, as well as for their invaluable help and advice. We also wish to thank Dr E. Katz from the Department of Virology, The Hebrew University-Hadassah Medical School, Jerusalem, for supplying us with HeLa cells, and Dr J. Hochman from the Department of Zoology, The Hebrew University of Jerusalem, for supplying us with S49 lymphoma cells.

References

[1] Levitzki, A., Atlas, D. and Steer, M. L. (1974) Proc. Natl. Acad. Sci. USA. 71, 2773–2776.
[2] Levitzki, A., Sevilla, N., Atlas, D. and Steer, M. L. (1975) J. Mol. Biol. 97, 35–53.
[3] Brown, E. M., Hauser, D., Trexler, F. and Aurbach, G. D. (1976) J. Biol. Chem. 251, 1232–1238.
[4] Mukherjee, C., Caron, M. G., Coverstone, M. and Lefkowitz, R. J. (1975) J. Biol. Chem. 250, 4869–4876.
[5] Williams, L. T., Snyderman, R. and Lefkowitz, R. J. (1976) J. Clin. Invest. 57, 149–155.
[6] Williams, L. T., Jarret, L. and Lefkowitz, R. J. (1976) J. Biol. Chem. 251, 3096–3104.
[7] Maguire, M. E., Wiklund, R. A., Anderson, H. J. and Gilman, A. G. (1976) J. Biol. Chem. 251, 1221–1231.
[8] Insel, P. A., Maguire, M. E., Gilman, A. G., Bourne, H. R., Coffino, P. and Melmon, K. L. (1976) Mol. Pharmacol. 12, 1062–1069.
[9] Harden, T. K., Wolfe, B. B., Johnson, G. L., Perkins, J. P. and Molinoff, P. B. (1976) Trans. Am. Soc. Neurochem. 7, 231.
[10] Brown, E. M., Gardner, J. D. and Aurbach, G. D. (1976) Endocrinol. 99, 1370–1376.
[11] Hanski, E. and Levitzki, A. (1977) Life Sci. in press.
[12] Atlas, D., Hanski, E. and Levitzki, A. (1977) Nature 268, 5014–5016.
[13] Tallman, J. F., Smith, C. C. and Henneberry, R. C. (1977) Proc. Natl. Acad. Sci. USA 74, 873–877.
[14] Atlas, D. and Levitzki, A. (1977) Proc. Natl. Acad. Sci. USA in press.
[15] Melamed, E., Lahav, M. and Atlas, D. (1976) Nature 261, 420–422.
[16] Melamed, E., Lahav, M. and Atlas, D. (1976) Brain Res. 116, 511–515.
[17] Melamed, E., Lahav, M. and Atlas, D. (1976) Experientia, 32, 1387–1389.
[18] Atlas, D., Melamed, E. and Lahav, M. (1977) Lab. Invest. 36, 465–470.
[19] Atlas, D., Reichberg, V. I. and Changeux, J. P. (1977) Brain. Res. 128, 532–536.
[20] Melamed, E., Lahav, M. and Atlas, D. (1977) Stroke 8, 261–263.
[21] Melamed, E., Lahav, M. and Atlas, D. (1977) Brain Res. 128, 379–384.
[22] Atlas, D. and Segal, M. (1977) Brain. Res. in press.
[23] Steer, M. L. and Levitzki, A. (1975) Arch. Biochem. Biophys. 167, 371–376.
[24] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[25] Yaffe, D. (1973) Tissue Culture, Vol. II, Preparation of Primary Cultures, pp. 106–114, Academic Press, New York.
[26] Wahrmann, A., Luzzati, D. and Winand, R. (1973) Biochem. Biophys. Res. Commun. 52, 576–581.
[27] Schubert, D., Tarikas, H. and La Corbiere, M. (1976) Science 192, 471–472.