IN VIVO LABELLING OF β-ADRENERGIC RECEPTORS ON RAT GLIOMA CELLS

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1. Introduction

Aurbach and colleagues [1,2] have recently developed an iodinated adrenergic antagonist [^{125}I]iodohydroxybenzylpindolol [^{125}IHYP) of very high specific activity which binds in a sterospecific manner to β-adrenergic receptors of partially purified membranes from turkey erythrocytes. The binding of ^{125}IHYP to membranes from rat heart [3], brain [4], human astrocytoma cells [5] and rat glioma cells [6] has also been reported. We have developed an in vivo assay for the binding of ^{125}IHYP to intact glioma cells. This method has many advantages over the in vitro assay and would allow the identification and direct localization of β-receptor on cells in tissue culture and tissue sections by autoradiography.

2. Materials and methods

2.1. *Growth of C6 rat glioma cells and binding of iodohydroxybenzylpindolol*

HYP (generously donated by Dr Hauser, Sandoz) was iodinated with ^{125}I as described by Maguire et al. [6]. Its specific activity was 600 Ci/mmol. C6 Rat glioma cells were grown on Falcon tissue culture plates in DMEM (Gibco) supplemented with 10% fetal calf serum (Gibco), under an atmosphere of 95% air, 5% CO_2. The cells in exponential-phase of growth were detached by trypsinization, distributed into 3.5 cm plastic Petri dishes and allowed to recover for 48 h in fresh medium before the binding assay. Each dish was washed twice with 5 ml PBS and incubated for 30 min. at 37°C, with 0.8 ml PBS (or serum, as indicated) containing 0.34 nM ^{125}IHYP (144 000 cpm), supplemented or not with $5 \cdot 10^{-5}$ M phentolamine. After incubation the medium was aspirated and the plates washed five times with 2 ml PBS at room temperature, followed by six more washings with 2 ml PBS, for 10 min periods. In the standard assay, PBS used for washing, was also supplemented with $5 \cdot 10^{-5}$ M phentolamine. Propranolol was present, as indicated, during the incubation of the cells with ^{125}IHYP. The cells were routinely detached from the plates, after washing with 1 ml 0.1% trypsin in D-1 isotonic buffer and counted in a gamma-counter. Detaching the cells from the dishes by scraping with a rubber policeman or by using trypsin 0.1% in D-1 isotonic buffer gives equal bindings properties (data not shown).

3. Results

In preliminary experiments 'specific' binding was defined as the percentage of ^{125}IHYP displaced when

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20 μM (±) propranolol was included in the binding-step. Only 20–30% 'specific' binding could be achieved when the plates were washed five times. Inclusion of 10% fetal calf serum or serum albumin (10 mg/ml) did not improve the efficiency of the washing procedure. The percentage of ^{125}IHYP specifically bound could be increased to 90% by further washing the plates 6 times, for 6 X 10 min periods, with PBS at room temperature. ^{125}IHYP non-specifically bound decreased linearly during these washing periods while the amount specifically bound remained constant. Brown et al. [2] and Maguire et al. [6] have observed dissociation of bound ^{125}IHYP only in the presence of competing cold HYP [2] or propranolol [6]. Prolonged washing is probably rendered necessary by the very high proportion of the ^{125}IHYP non-specifically bound to the membrane (70% of the load). Saturation of the binding sites could, however, not be observed under these conditions. As shown in fig.1, inhibition of binding by (–) propranolol is biphasic. Only inhibition at low concentrations (up to 10^{-7} M) of propranolol reflects binding to adrenergic receptors. At higher concentrations, the inhibition is not stereo-specific and affects non specific sites. Similar observations have been made in vitro in the C6 glioma cell-line [6] and in rat brain [4]. Specific binding was thus redefined as the decrease in bound ^{125}IHYP observed when the cells were coincubated with 0.1 μM (±) propranolol. As shown in fig.2 a saturable binding curve was obtained in this way.

In order to decrease the percentage of ^{125}IHYP bound non-specifically, two approaches were undertaken. Cells were incubated with ^{125}IHYP in PBS containing varying concentrations of fetal calf serum. There is a proportional decrease of the binding to non-specific sites with increasing concentrations of serum. Therefore it is reasonable to consider a partition of the non-specifically bound ^{125}IHYP between the membranes and serum components compare with the partition of cholesterol between plasma high density lipoprotein [14,15] and arterial smooth-muscle

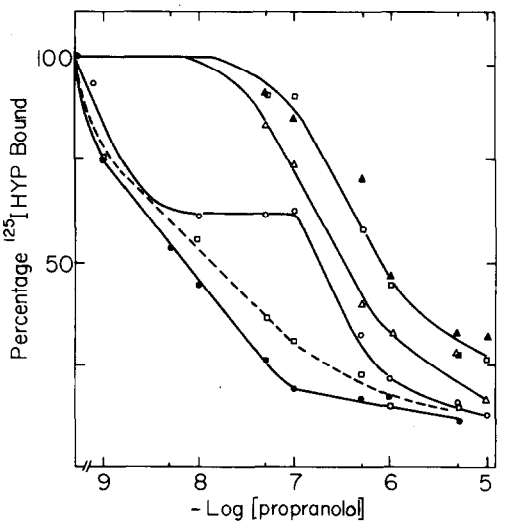

Fig.1. Inhibition of ^{125}IHYP-binding to C6 cells by propranolol. (a) Loading of the cells in PBS alone, competition with (–) propranolol (–○–○–) or (+) propranolol (–△–△–). (b) Loading of the cells in 100% fetal calf serum, competition with (–) propranolol (- -□- -) or (+) propranolol (-□-□-). (c) Loading of the cells in PBS + $5 \cdot 10^{-5}$ M phentolamine, competition with (–) propranolol (–●–●–) or (+) propranolol (–▲–▲–). 100% Binding refers to the value obtained in absence of competing propranolol. The presence of phentolamine during the washing steps did not further decrease the non-specific binding of ^{125}IHYP to the cells but was essential in order to remove all the ^{125}IHYP trapped in the plastic plate, that could give a high background in autoradiography experiments. ^{125}IHYP did not bind so tightly to glass and could be removed by washing with PBS alone.

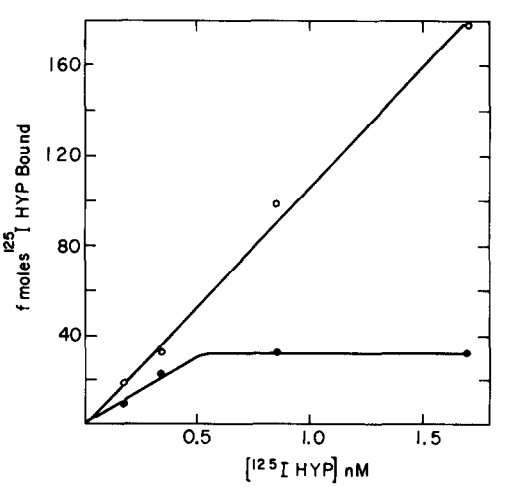

Fig.2. Saturation of ^{125}IHYP-binding in PBS. $3.1 \cdot 10^6$ Cells (1 mg protein) were incubated with various concentrations of ^{125}IHYP in PBS, and washed as indicated in fig.1. (–●–●–) ^{125}IHYP specifically bound, displaced by 0.1 μM DL-propranolol (–○–○–) ^{125}IHYP non-specifically bound.

cells). Furthermore the high hydrophobicity of HYP favors its solubility in the lipid-phase of the membrane. It must be noted that different types of cells bind ^{125}IHYP non-specifically to a different extent. This may be related to the fluidity of their membrane. Brown et al. have recently reported [13] and we have independently observed (data not shown) that a much smaller percentage of ^{125}IHYP is non-specifically bound to intact erythrocytes compared to C6 glioma cells. We have also observed (results unpublished) that at stationary phase, the extent of non-specifically bound ^{125}IHYP increases. As shown in fig.1 the displacement curve of ^{125}IHYP in the presence of (–) propranolol, with the cells loaded in the presence of 100% fetal calf serum is no longer biphasic, indicating the suppression of the high percentage of binding to non-specific sites. However a clear saturation of specific ^{125}IHYP binding sites could not be achieved by loading the cells in 100% fetal calf-serum.

Therefore a standard assay was developed based on the report by Sporn and Molinoff [4] that phentolamine could markedly reduce non-specific binding to membranes from cerebral cortex, caudate nucleus and cerebellum. Phentolamine at concentrations of 0.1–0.05 mM eliminated the in vivo binding of ^{125}IHYP to non-specific sites in C6 glioma cells, as shown in fig.1 and fig.3. In the presence of phentolamine, (–) propranolol was a more potent inhibitor of the ^{125}IHYP binding, by 2 orders of magnitude, than the corresponding (+) isomer. The K_D of ^{125}IHYP determined by Scatchard analysis of the data of fig.3 was $1.4 \cdot 10^{-10}$ M which compares to similar values obtained in vitro [6]. From fig.3 it was calculated that there were 7000 β-adrenergic receptors/C6 glioma cell. This value was very reproducible and did not vary with the phase of growth of the cells (results not shown). The enhanced sensitivity of glioma or astrocytoma cells to catecholamines in the stationary-phase of growth [7] is not caused by an increase in the number of β-adrenergic receptors. Our value of 7000 receptors/cell is higher than the number obtained in vitro (4000) [6], probably due to partial loss or damage of the plasma membranes during their preparation. It can be concluded from these results that the properties of the in vivo binding of ^{125}IHYP are qualitatively similar to those obtained in vitro in a membrane preparation. However, the in vivo assay is more physiological and should allow the determination of the spatial distribution of the β-receptors by autoradiography.

Incubation of the C6 cells with the β-agonist, isoproterenol was associated with a net loss of receptors, as shown in fig.4, 50% of the ^{125}IHYP binding capacity of the intact cells was lost after 2 h of exposure to isoproterenol. This observation suggests that the disappearance or inactivation of the β-adrenergic receptors could be one of the mechanisms responsible for the desensitization phenomenon described earlier

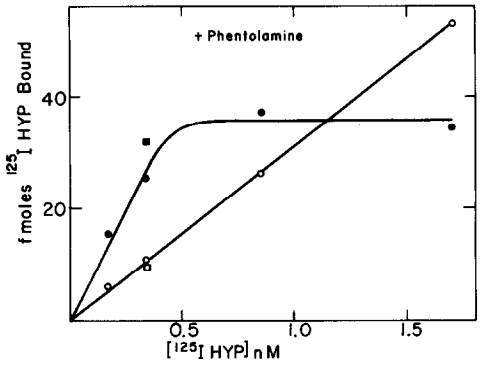

Fig.3. Saturation of ^{125}IHYP-binding in PBS + phentolamine. $3.1 \cdot 10^6$ Cells were incubated with ^{125}IHYP in PBS + $5 \cdot 10^{-5}$ M. (■,□) or 10^{-4} M (●,○) phentolamine and washed as indicated in fig.1. (–●–●–) ^{125}IHYP Specifically bound, displaced by 0.1 μM DL-propranolol. (–○–○–) ^{125}IHYP Non-specifically bound.

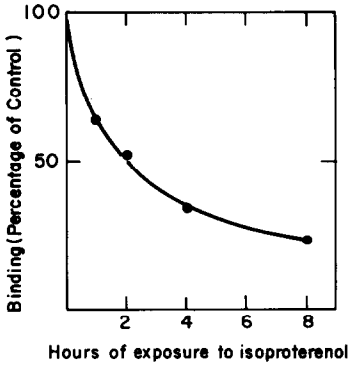

Fig.4. Loss of ^{125}IHYP-receptors after incubation with isoproterenol. Cells were incubated with 10^{-4} M DL-isoproterenol in DMEM supplemented with 10% serum, at 37°C, for various periods. β-Adrenergic receptors were then quantified as described in fig.3.

in the C6 cell-line [8–11]. A similar observation has been made in frog erythrocytes [12]. Partial reversal of catecholamine refractoriness has also been obtained by inhibitors of RNA and protein synthesis acting at the level of phosphodiesterase [8] or elsewhere [8,10,11]. These results, taken together, indicate that desensitization to catecholamines may be due to at least two independent mechanisms. However, there is no clear relationship between desensitization and morphological changes. Serum removal, which increases cyclic AMP levels [7] and induces a marked change in the morphology of the cells does not affect their number of β-adrenergic receptors.

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