Volume 233, number 2, 352–354 FEB 05968 June 1988

1,4-Dihydropyridine receptor associated with Ca^{2+} channels in human embryonic fibroblasts

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Received 31 March 1988

By using the radioactively labeled 1,4-dihydropyridine (DHP) probe, [^{3}H]PMD, we have demonstrated that cultured human embryonic fibroblasts grown at a low density in Eagle's medium supplemented with serum contain a single class of non-interacting DHP binding sites (B_{max}, 1.2 ± 0.3 pmol/10^6 cells; K_d, 3.9 nM). After inhibition of the DHP receptor biosynthesis by cycloheximide, the number of [^{3}H]PMD binding sites is reduced with a half-time of 12 h, which implies a turnover rate of 30000 ± 7500 receptors/h per cell. With progression to confluency, the B_{max} value decreased up to 0.28 ± 0.08 pmol/10^6 cells without significant change in K_d value. When cells were grown at a low density in serum-free conditions, the number of [^{3}H]PMD binding sites gradually increased 1.9-fold within 3 days. Addition of serum reversed this effect with the same time course. These results imply that the DHP-sensitive Ca^{2+} channels are involved in the control of the proliferation of human embryonic fibroblasts.

Dihydropyridine receptor; Ca^{2+} channel; Proliferation; (Human embryonic fibroblast)

1. INTRODUCTION

The 1,4-dihydropyridine Ca^{2+} entry blockers have proved most useful as high-affinity probes for the long-lasting, or so-called L-type, Ca^{2+} channels [1,2]. These channels are involved in the regulation of Ca^{2+}-dependent functions in a variety of cells. The depolarization of plasma membrane induced by various physiological stimuli results in the opening of Ca^{2+} channels which, in turn, leads to the transient rise in the cytoplasmic Ca^{2+} concentration. On the other hand, intracellular free Ca^{2+} plays a key role in the control of a variety of cellular functions, including secretion [3], proliferation [4], etc. Thus, a detailed knowledge of Ca^{2+} channel functioning is a prerequisite for the insight into the intimate mechanisms of the cell growth regulation.

The present experiments are undertaken to identify directly the DHP receptor in cultured human embryonic fibroblasts and to define whether or not this receptor associated with Ca^{2+} channels is under the control of the cell growth conditions.

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Abbreviations: BSA, bovine serum albumin; DHP, 1,4-dihydropyridine; HEF, human embryonic fibroblast; [^{3}H]PMD, 2,6-dimethyl-3-methoxycarbonyl-5-([2,3-$^3H_2$]-*n*-propoxycarbonyl)-4-(2′-difluoromethoxyphenyl)-1,4-dihydropyridine; B_{max}, maximum receptor density; K_d, receptor dissociation constant

2. MATERIALS AND METHODS

2.1. *Chemicals*

Standard reagents were obtained from Serva. D-*cis*-Diltiazem was purchased from Goedecke. [^{3}H]PMD enriched to 98% (60–62 Ci/mmol) was synthesized in this laboratory. Nitrendipine was kindly provided by Dr G.J. Dubur (Institute of Organic Synthesis, Riga).

2.2. *Cells*

HEFs were prepared as described [5] and grown in 6-macrowell plates (Linbro) in Eagle's medium supplemented with 8% calf serum and 2% foetal calf serum (Flow). 4th to 15th passage cells were used in experiments. Serum-deprived cells were grown in Eagle's medium containing 0.1% BSA. The turnover rate of the DHP receptor was measured after inhibi-

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tion of protein synthesis by 10^{-4} M cycloheximide added to standard serum culture medium.

2.3. *[^{3}H]PMD binding studies*

Culture medium was removed, cells attached to plates were rapidly washed twice with 2 ml of incubation buffer (20 mM Tris-HCl, pH 7.35, 146 mM NaCl, 1 mM $CaCl_2$, 10 μM D-*cis*-diltiazem). Diltiazem was used throughout the experiments to enhance the affinity of [^{3}H]PMD to DHP receptors [6]. The assays were incubated with radioactive probe at 22°C for 1 h in a total volume of 2 ml of incubation medium. In determining saturation binding, the concentration of [^{3}H]PMD was varied between 1.0 and 12 nM. Incubations were terminated by aspiration of the medium, cells were rapidly washed twice with 2 ml of incubation buffer and dissolved in 1 ml of 0.4 M NaOH containing 2.5% SDS. 0.5-ml aliquots were counted for radioactivity in Ready-Solv EP scintillation cocktail (Beckman). The rest of the samples were saved for protein determination according to Hartree method [7]. Specific binding of [^{3}H]PMD was defined as the difference between total radioactivity bound and non-specific binding determined in the presence of 1 μM nitrendipine. At a radioligand concentration of 2 nM, the non-specific binding amounted to 40% of the total [^{3}H]PMD binding. Each value was measured as a mean of 3–6 determinations. The adsorption of [^{3}H]PMD by the plates was negligible.

The time course of the changes in DHP receptor content was followed at [^{3}H]PMD concentration of about 2 nM.

3. RESULTS AND DISCUSSION

The binding of [^{3}H]PMD, a novel highly specific DHP probe [8], was used in these experiments as a biochemical measurement of DHP-sensitive Ca^{2+} channels in cultured HEFs. Equilibrium binding studies made with HEFs grown at low density in Eagle's medium supplemented with serum, revealed a single class of non-interacting [^{3}H]PMD binding sites (fig.1). The B_{max} and K_d values are 1.2 ± 0.3 pmol/10^6 cells and 3.9 nM, respectively. The K_d value obtained is similar to the one (3.2 nM) determined previously for skeletal muscle [^{3}H]PMD receptor [8]. The result is in line with the recently reported data of Chen and Hess [9] who observed L-type Ca^{2+} channels in human fibroblasts by the patch clamp technique.

The turnover rate of the DHP receptor was estimated from measurements of the rate of disappearance of [^{3}H]PMD binding sites after inhibition of protein synthesis by cycloheximide (fig.2). The loss of [^{3}H]PMD binding is approximately exponential with a half-life of 12 h. This corresponds to a turnover rate of 30000 ± 7500 DHP receptors/h per cell.

The content of the DHP receptors significantly depends on the density of cells in culture. The

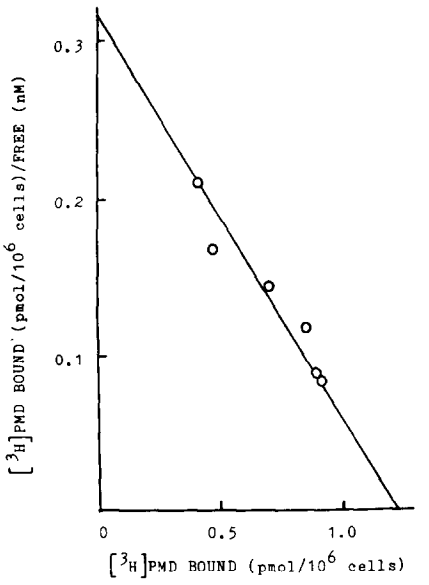

Fig.1. Scatchard plot for the specific binding of [^{3}H]PMD to cultured HEFs. Cells were grown in a standard serum medium at a density of 5–7 × 10^3 cells/cm^2. 10^6 cells correspond to 0.41 mg protein.

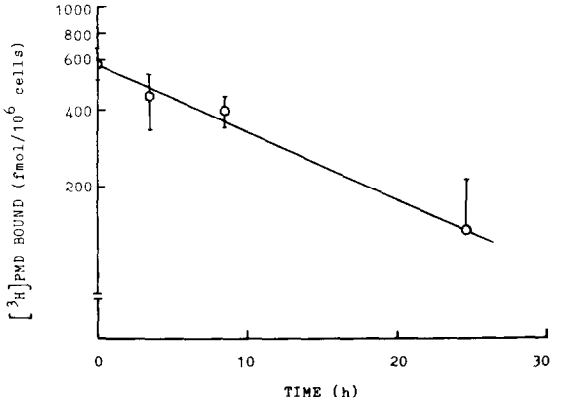

Fig.2. Turnover rate of the DHP receptor in cultured HEFs. Cells were grown at a density of 5–7 × 10^3 cells/cm^2 in a standard serum medium containing 10^{-4} M cycloheximide. Changes in [^{3}H]PMD binding site number were measured at a radioligand concentration of 1.7 nM.

above-mentioned results were obtained for cells grown at densities less than 7000 cells/cm^2, when practically no multiple cell-cell contacts were observed. With progression to confluency, the specific binding of [^{3}H]PMD decreased up to 0.2–0.3 pmol/10^6 cells (fig.3) without significant change in the K_d value. Therefore, the content of

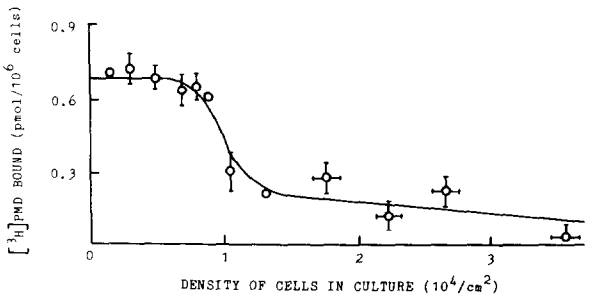

Fig.3. Dependence of [^{3}H]PMD binding to HEFs on cell density in cultures. Changes in [^{3}H]PMD binding capacity were measured at a radioligand concentration of 2 nM.

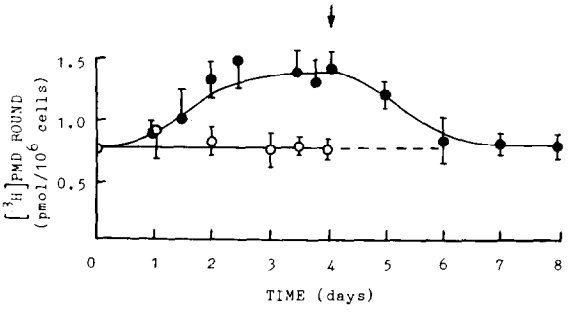

Fig.4. Effect of serum on DHP receptor levels in HEFs. Cells were grown in a standard serum medium at a density of 3–5 × 10^3 cells/cm^2. On day 2, the growth medium was replaced with serum-free Eagle's medium supplemented with 0.1% BSA (•) or standard serum medium (○). Arrow indicates the time at which standard serum medium was added to serum-deprived cells. The time course of changes in [^{3}H]PMD binding level was measured at a radioligand concentration of 2 nM.

the DHP receptor binding sites is affected by cell-cell interactions and the related cessation of cell growth.

In cultured HEFs maintained in subconfluent state the elimination of serum from culture medium induces the increase in [^{3}H]PMD binding capacity which attains a steady-state level (B_{max}, 2.3 ± 0.7 pmol/10^6 cells; K_d, 3.2 nM) within 3 days (fig.4). This effect was completely reversible: when serum-deprived cells were grown further on standard serum medium, the [^{3}H]PMD binding capacity reapproached the initial level with about the same time course (fig.4). The changes observed occur with a characteristic rate of the receptor turnover (about 1.1 pmol of DHP binding sites per 10^6 cells every 3 days) detectable at the radioligand concentration used, and therefore, most probably are maintained at the level of translation.

So, one can conclude that the slow-down of proliferation induced either by the formation of confluent monolayer or by deprivation of serum from culture media of low cell density leads to opposite changes in the number of DHP receptor binding sites. Taken together, these results imply that the DHP receptors associated with Ca^{2+} channels are involved in the regulation of HEF proliferation. It would be of interest to investigate whether Ca^{2+} channels contribute to mitogenic activation of quiescent fibroblasts.

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