Growth-related variation of α_2-adrenergic receptivity in the HT 29 adenocarcinoma cell-line from human colon

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The human colon adenocarcinoma cell-line HT 29 has been shown to possess functional α_2-adrenergic receptors. Here, [^{3}H] clonidine was used as radioligand to study the evolution of α_2-adrenergic receptivity during the time course of HT 29 cell culture. Scatchard analysis of the saturation curves indicates that the number of [^{3}H]clonidine binding sites increases throughout the 17 day culture period. The maximal number of α_2-adrenoceptors is found during the stationary phase of growth, when cell density is high and mitotic rate low. Moreover, the use of adrenaline and clonidine as α_2-adrenergic agonists reveals a relationship between the number of receptors and the intensity of the biological effect associated with their stimulation (inhibition of the VIP-induced cyclic AMP accumulation).

α_2-Adrenoceptor *HT 29 cell-line* *[^{3}H]Clonidine* *Cyclic AMP* *Catecholamines* *Cell culture*

1. INTRODUCTION

During the last decade, the hormone receptor-adenylate cyclase coupling system appeared to be more complex and dynamic than originally believed. Indeed, several lines of evidence have indicated that the changes in adenylate cyclase activity in response to agonist exposure could be regulated by different factors, e.g., the number of membrane receptors on target cells, the nucleotide requiring proteins which couple the receptor to the cyclase, the adenylate cyclase itself and the physicochemical properties of the membrane (review [1]).

This view mainly results from studies carried out on β-adrenergic receptors. By contrast, only scanty information is available concerning the regulation of α_2-adrenergic receptivity. In [2], we described the characteristics of α_2-adrenoceptors on the adenocarcinoma cell HT 29 derived from human colon mucosa. This receptor, which is negatively coupled to the adenylate-cyclase system [3], could be advantageously studied in such a model for better understanding the α_2-adrenoceptor regulation. This work was undertaken to study the evolution of HT 29 cell α_2-adrenoceptor equipment as a function of the cell culture time course. We found: (i) that the number of α_2-adrenoceptors per mg cell membrane protein is a function of cell density and (ii) that there is a close relationship between the number of α_2-adrenoceptors on the cell and the intensity of the biological effect associated with their stimulation (inhibition of the VIP-induced cyclic AMP accumulation).

2. MATERIALS AND METHODS

2.1. *Materials*

The following chemicals were purchased from the indicated sources: [^{3}H]clonidine (spec. act. 30 Ci/mmol) from Amersham; adrenaline bitartrate, bacitracin, 3-isobutylmethylxanthine (IBMX), ascorbic acid and EDTA from Sigma; clonidine hydrochloride from Boehringer-Ingelheim and the trypsin solution (2.5%) from Gibco. Vasoactive intestinal peptide (VIP) was a generous gift from Dr M. Laburthe (Paris).

2.2. *Cell culture*

The HT 29 cell-line has been established in permanent culture by Dr J. Fogh, from a human co-

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lon carcinoma [4]. The cells were subcultured in 22-cm^2 plastic Petri dishes (Falcon, France), as described in [3]. Unless otherwise stated, cell seeding was done from post-confluent HT 29 cells as follows: the cells were detached by incubating the cell layer for 5 min at 37°C in a phosphate buffer solution containing 0.25% trypsin and 0.5 mM EDTA. The cells were then collected by centrifugation (800 × g, 10 min), resuspended in fresh culture medium, seeded at a density of 2.5×10^4 cells/cm^2 and grown under an air/5% CO_2 controlled atmosphere at 37°C. The culture medium was changed each 48 h and all experiments were done 24 h after the last medium change.

Protein content in the monolayer has been reported to be linearly correlated with the number of cells per dish [4]. To verify this point under our experimental conditions, trypsinized detached cells were directly counted under a microscope. The results indicated that 1 mg protein represents 3.1×10^6 cells at any stage of the culture and that protein content per dish can be used as an index of cell growth.

2.3. *Cell membrane preparation and binding studies*

Membrane preparation was performed as in [3]; protein content in the 'membrane preparation' was found to represent about 38% (range 36–41%) of the total cell proteins at all stages of the experiment. The saturation assays of α_2-adrenoceptors were done according to [5], slightly modified as follows; to determine the total binding, 100 μl crude membrane preparation (2–3 mg protein/ml) were incubated with 300 μl Tris-buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5) containing [^{3}H]clonidine at appropriate concentrations (0–10 nM). Non-specific binding was determined in parallel assays containing 10 μM unlabelled clonidine. After 15 min at 37°C, the incubation was stopped by the addition of 4 ml ice-cold Tris buffer. The filters were dried and counted in a β-counter. The binding parameters (B_{max} and K_D) were determined from Scatchard analysis [6]. All binding experiments were performed at least twice.

2.4. *α_2-Adrenoceptor stimulation and measurement of cyclic AMP levels*

Four h before starting the experiments, the standard culture medium was replaced by 4 ml serum-free medium to avoid interference with fetal calf serum components. Receptor stimulations were done on intact attached cells by adding 40 μl drug and/or hormone solution at the appropriate concentration in the presence of 0.5 mM IBMX and 0.2% bacitracin. When adrenaline was tested, 0.75 mM ascorbic acid was added to prevent oxidation and 50 μM propranolol was added to avoid interference with β-adrenoceptors. After 15 min at 37°C, the medium was removed and the incubation rapidly stopped by addition of 4 ml of 95% methanol/5% formic acid mixture. Cyclic AMP levels were measured in the evaporated methanol extracts by the radioimmunological method [7] using the RIA kit from New England Nuclear. All reported values refer to means ± SE of 4 separate experiments.

Protein content was determined using the brilliant blue method [8] and bovine serum albumin as standard.

3. RESULTS

3.1. *Evolution in the number of [^{3}H]clonidine binding sites*

Fig.1 shows the evolution pattern of total protein content and of the number of [^{3}H]clonidine binding sites during HT 29 cell culture. The protein content curve, which reflects the cell growth, shows the two classical phases: the exponential phase (day 3 to 11) where cell divisions are rapid and the stationary phase (day 12 to 17) where the rate of cell division slackens. The determination of the maximum number of [^{3}H]clonidine binding sites at saturating concentration of the ligand indicates that the number of α_2-adrenoceptors expressed per mg cell membrane protein increases during the time course of the cell culture. This increase is especially rapid during the exponential phase (day 3 to 11) when the number of [^{3}H]clonidine binding sites rises from 14 to 119 fmol/mg membrane protein. At the last stage of the culture, the number of clonidine binding sites reaches a plateau around 130 fmol/mg membrane protein. Since these experiments were done on membrane preparations, it is important to ensure that the observed increase in receptor number is not artefactual but reflects changes in the intact cell. As membrane proteins are regularly found to represent about 38% of total cell proteins, it can be

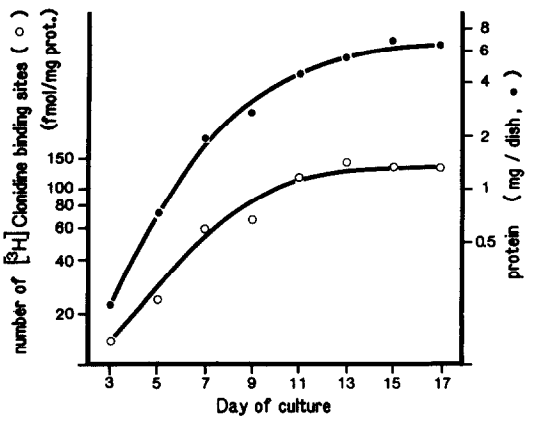

Fig.1. Evolution of α_2-adrenoceptor number in the course of HT 29 cell growth in culture. The cells were seeded at a density of 2.5×10^4 cells per cm^2. Measurements were performed as described in section 2 on days 3, 5, 7, 9, 11, 13, 15 and 17 of culture. The total protein content expressed as mg per flask (●—●) and the number of [^{3}H]clonidine binding sites expressed as fmol per mg cell membrane protein (○—○) are represented on log scales. The given values are means of 2 different experiments.

Table 1

Effects of EDTA or EDTA + trypsin treatment on [^{3}H]clonidine binding parameters

	B_{max} (fmol/mg membrane protein)	K_D (nM)
Batch A, control	129	1.05
Batch B, 0.5 mM EDTA	121	1.02
Batch C, 0.5 mM EDTA + 0.25% trypsin	156	1.01

Three batches of HT 29 cells were prepared, as indicated in the text, from attached cells (batch A) or from isolated cells obtained by treatment of the cell layer with 0.5 mM EDTA (batch B) or 0.5 mM EDTA + 0.25% trypsin (batch C). Binding parameters were calculated from Scatchard analysis

calculated that the number of binding sites, expressed on a per cell basis, increases from 1010 (day 3) to 11200 (day 15). By contrast, it must be pointed out that the affinity of the adrenoceptors for the radioligand remains roughly constant (K_D = 0.84–1.46 nM) during the entire culture period.

Since the petri dishes were seeded with trypsinized cells from a 12-day-old cell layer, further experiments were designed to determine whether the action of trypsin was responsible for the loss of clonidine binding sites and, furthermore, to understand better the determinism of α_2-receptivity appearance. In these experiments, 3 batches of crude membrane preparations were obtained from post-confluent cells (day 14) as follows: the first batch (A = control) was prepared from an attached frozen cell layer using neither trypsin nor EDTA, while batches B and C were obtained from frozen cells previously isolated by incubating the cell layer 5 min at 37°C in the presence of 0.5 mM EDTA or 0.5 mM EDTA + 0.25% trypsin, respectively. The [^{3}H]clonidine binding parameters for these 3 preparations, which are reported in table 1, clearly indicate that trypsinization was not responsible for the decrease in the number of α_2-receptors observed after seeding.

In the experiments reported in table 2, HT 29 trypsinized cells obtained from a 14-day-old cell layer were seeded at densities of 0.5, 1.0, 4.0 or 8.0×10^6 cells per dish. Three days after seeding, the cell layers were frozen and membranes were routinely prepared; the binding study indicates that α_2-adrenoceptors are still present on high density seeded cells while nearly all are lost on low density seeded cells. Moreover, further results show that there is a good correlation between the number of clonidine binding sites and the protein content in the cell layer, irrespective of the dura-

Table 2

Effect of cell density seeding on [^{3}H]clonidine binding site number

Seeding density (10^6 cells/dish)	Total protein (mg/dish)	B_{max} (fmol/mg membrane protein)	K_D (nM)
0.5	0.22	14	1.2
1	0.39	44	1.2
4	1.75	90	1.4
8	3.05	140	1.6

The HT 29 cells were seeded at densities of 0.5, 1, 4 or 8×10^6 per dish. After 3 days of culture, membranes were prepared and [^{3}H]clonidine binding parameters determined from Scatchard analysis

tion of the culture (not shown). Such results indicate that the presence of α_2-adrenoceptors on HT 29 cells is not time dependent, but rather is a function of cell density in the petri dish.

3.2. *Evolution of the intensity of the biological effect*

The last part of this work was carried out to determine whether the variation in the number of clonidine binding sites during the culture period was correlated with changes in the intensity of the α_2-receptor mediated biological effect. In these experiments, the effects of 10 μM adrenaline (plus 50 μM propranolol) or 10 μM clonidine on the accumulation of cyclic AMP induced by 5 nM VIP were studied. Under our experimental conditions, this VIP concentration was found to increase the cyclic AMP level from 8 ± 2 to 1120 ± 97 pmol/mg cell proteins (not shown) which corresponds to a submaximal stimulation [3]. The stimulations were done as described in section 2 on the same batch of dishes as those used for binding assays. The intensity of the biological effect, expressed as percent of inhibition of cyclic AMP accumulation as a function of the number of receptors is reported in fig.2. For both adrenaline and clonidine, it appears that there is a close relationship between the inhibition rate and the number of receptors at different stages of the culture. The increasing inhibitory effect is not due to a decrease of VIP efficiency since the cyclic AMP accumulation induced by this polypeptide was fairly constant during the whole culture period (not shown).

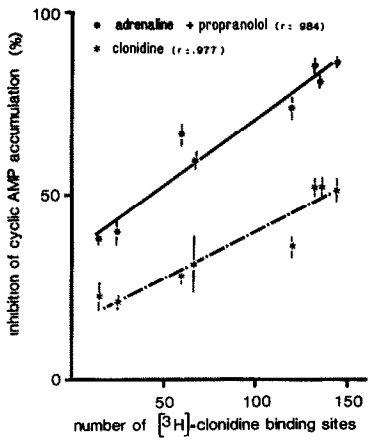

Fig.2. Evolution of the intensity of α_2-agonist biological effects as a function of HT 29 cell membrane α_2-adrenoceptor number. The effects of 10 μM clonidine and 10 μM adrenaline (plus 50 μM propranolol) were tested on the cyclic AMP accumulation induced by 5 nM VIP. Results are expressed as percent of inhibition of cyclic AMP accumulation. Reported values are means ± SE from 4 different experiments.

Besides, when comparing the biological potencies of clonidine and adrenaline in fig.2, it is found that, as previously noted [3], clonidine is only a partial agonist.

Finally, it must be pointed out that there is no direct proportionality between the number of receptors and the intensity of the associated biological effect; the biological effect of clonidine or adrenaline at day 3 (22 and 38% inhibition, respectively) is half that found at day 17 (52 and 86%) while the receptor number is 10-times lower (14 vs 130 fmol/mg membrane protein).

4. DISCUSSION

In [2,3] we showed the presence of α_2-adrenoceptors on HT 29 cancer cells. This cell-line, established in permanent culture, was believed to be a good model for studying the regulation of the α_2-adrenergic receptivity as a function of the hormonal and/or nutritional conditions of the cell culture. At present, very little is known about the regulation of this class of receptor. Both in vitro and in vivo experiments on platelets [9,10], liver [11] or adipocytes [12,13] have shown that, unlike β-receptors, α_2-adrenoceptors are not down-regulated after agonist exposure. However, several reports showed that cell α_2-receptivity can vary to a large extent: for instance, study of the ontogeny of α-adrenoceptors in rat brain indicated that [^{3}H]clonidine binding rate increases rapidly during the first weeks after birth. Similarly, Carpéné et al. [15] reported a huge increase of the α_2-adrenergic responsiveness of hamster adipocytes during growth and fattening; moreover, it was shown that starvation and depletion of the fat stores in this animal induced the loss of α_2-adrenoceptors and the disappearance of the associated antilipolytic effect.

Our results indicate that, in HT 29 cells, there is a significant increase in the number of α_2-receptors during the time course of the culture; furthermore, we found that the receptor number is a function of

cell density. Unlike what was reported concerning platelets from patients with major depressive disorders [16], the increase of [^{3}H]clonidine binding sites in HT 29 cells is not due to a change in the affinity state of the receptor; indeed, the use of the tritiated antagonist, yohimbine, for labelling HT 29 cell α_2-adrenoceptors indicated a similar evolution pattern for the yohimbine binding sites (unpublished). Our work also clearly shows that there is a relationship between the number of receptors and the intensity of the biological effect associated to their stimulation in our model.

At present, we have no explanation for the α_2-adrenoceptor maturation during the culture. Among the different possible mechanisms, however, one can propose that the α_2-receptivity could vary according to the phases of the cell cyle, as is the case for β-adrenoceptors in rat glioma C6 cells [17]. It can be also hypothesized that membrane contact might be the major determinant of α_2-receptor biosynthesis. These two proposals are under investigation.

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REFERENCES

[1] Lefkowitz, R.J., Stadel, J.M. and Caron, M.G. (1983) Annu. Rev. Biochem. 52, 159–186.
[2] Carpéné, C., Paris, H., Cortinovis, C., Viallard, V. and Murat, J.C. (1983) Gen. Pharmacol. 14, 701–703.
[3] Bouscarel, B., Cortinovis, C., Carpéné, C., Murat, J.C. and Paris, H. (1985) Eur. J. Pharmacol. 107, 223–231.
[4] Fogh, J., Wright, W.C. and Loveless, J.D. (1977) J. Natl. Cancer Inst. 58, 209–214.
[5] Berlan, M. and Lafontan, M. (1980) Eur. J. Pharmacol. 67, 481–484.
[6] Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660–672.
[7] Steiner, A.L., Parker, C.V. and Kipnis, D.M. (1982) J. Biol. Chem. 247, 1106–1112.
[8] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[9] Karliner, J.S., Motulsky, H.J. and Insel, P.A. (1982) Mol. Pharmacol. 21, 36–43.
[10] Motulsky, H.J., O'Connor, D.J. and Insel, P.A. (1983) Clin. Sci. 64, 265–272.
[11] Snavely, M.D., Mahan, L.C., O'Connor, D.J. and Insel, P.A. (1983) Endocrinology 113, 354–361.
[12] Pecquery, R., Leneuveu, M.C. and Guidicelli, Y. (1984) Endocrinology 114, 1576–1583.
[13] Villeneuve, A., Carpéné, C., Berlan, M. and Lafontan, M. (1985) J. Pharmacol. Exp. Ther., in press.
[14] Morris, J.M., Dausse, J.P., Devynk, M.A. and Meyer, P. (1980) Brain Res. 190, 268–271.
[15] Carpéné, C., Berlan, M. and Lafontan, M. (1983) J. Lipid Res. 24, 766–774.
[16] Garcia-Sevilla, J.A., Garcia-Vallejo, P. and Guimon, J. (1983) Eur. J. Pharmacol. 94, 359–360.
[17] Charlton, R.R. and Venter, J.C. (1980) Biochem. Biophys. Res. Commun. 94, 1221–1226.