MOLECULAR PHARMACOLOGY, 12, 362-372

Cell Density and Receptor-Adenylate Cyclase Relationships in the C-6 Astrocytoma Cell

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(Received July 14, 1975)

SUMMARY

MORRIS, STEPHEN A. & MAKMAN, MAYNARD H. (1976) Cell density and receptor-adenylate cyclase relationships in the C-6 astrocytoma cell. *Mol. Pharmacol.*, 12, 362-372.

The maximum stimulation at saturating concentrations of epinephrine (V_{max}) of catecholamine-sensitive adenylate cyclase of rat C-6 astrocytoma cells in culture is significantly enhanced when the cells achieve confluence. This relationship is apparent whether measured as increased levels of adenosine cyclic 3',5'-monophosphate (cAMP) in intact cells, without or with serum present, or as ATP-dependent cAMP formation by cell homogenates. Corresponding to this increase, the adenylate cyclase activity stimulated by fluoride increased with increased cell density, as was also noted for cAMP phosphodiesterase. When intact cells in the presence of serum are exposed to epinephrine for 2 min, the epinephrine concentration giving half-maximal stimulation (K_m) increases from 100 to 600 nM as cell cultures grow from low density to confluence; similarly, an increase in the dissociation constant K_b for the antagonist propranolol, from 1 to 10 nM, was found as cell density increased. The attribution of those trends to intercellular interaction rather than to total cell number per plate or to manipulation of cells at the time of subculture is supported by the absence of such trends in nonhomogeneously dispersed cell culture preparations. Thus, under these conditions of cell culture and investigation, the effective affinity for catecholamine or antagonist decreases as a function of cell density. It was further noted, however, that in the absence of serum the K_m for epinephrine did not change with cell density, and after cells had been incubated with propranolol for 45 min the apparent K_b was 1 nM, independent of cell density. It is concluded that the increase in V_{max} with increasing cell density is not associated with any inherent change in the affinity or sensitivity of the catecholamine receptor-cyclase complex, but may be related to an increase in total catalytic activity.

INTRODUCTION

A positive correlation between the catecholamine-stimulated adenylate cyclase activity of whole cells and increasing cell density has been reported for WI-38 fibroblasts (nontransformed) (1) and the rat astrocytoma C-6 line (transformed) (2). In contrast, using whole cells, an inverse correlation has been reported with the cloned tumor astrocyte line 1181N1 (3). Conflicting results with the earlier studies on WI-38 cells (1) have also been reported (4).

In the studies reported here, a transformed cell line, the rat astrocytoma C-6 cell, was used to re-examine the cell den-

This work was supported by Grants 5T5 GM 167411 and 5 R01 CA 13176 from the National Institutes of Health. A preliminary report of these findings was presented at the 11th Annual Pathobiology Seminar at the Given Institute of Pathobiology, Aspen, Colorado, July 13, 1974.

sity phenomenon as it affects catecholamine-sensitive adenylate cyclase in the intact cell. These studies sought to determine the possible contribution to the phenomenon of changes in either (a) the affinity of the catecholamine receptor linked to adenylate cyclase, (b) the sensitivity of the receptor-cyclase complex, or (c) the activities of the subcellular components contributing to the whole-cell phenomenon, i.e., the adenylate cyclase that is stimulated by fluoride and epinephrine, the cAMP[1] phosphodiesterase, or the manipulation and seeding of cells prior to subculture. In this regard, determinations were made for V_{max}, defined as the amount of cAMP accumulated in the presence of maximally stimulating concentrations of epinephrine in whole cells (with and without 10% calf-fetal calf serum) and in homogenates; for K_m, defined as the concentration of epinephrine at which half the maximum activity (V_{max}) occurs; for K_b, defined as the dissociation constant for propranolol; and for variations in the activity of both the cAMP phosphodiesterase and the adenylate cyclase that is stimulated by fluoride. Nonheterogeneously dispersed subcultures were analyzed in the same manner.

MATERIALS AND METHODS

Culture conditions. Cultured rat astrocytoma cells of the C-6 line were originally derived from glial tumors induced in Wistar rats with *N*-nitrosomethylurea (5). The medium used was that of Dulbecco, supplemented with 5% calf serum and 5% fetal calf serum, antibiotics as previously described (6), and Fungizone. Cells were grown as monolayers on 60-mm Falcon plastic tissue culture dishes in an atmosphere of 95% air and 5% CO_2. When the parent plates achieved full confluence, the cells were subcultured after incubation in 0.25% trypsin and 0.04% sodium EDTA, followed by gentle vortex-dispersion. Except where noted, experiments were carried out under the same conditions used for growth and 10 hr after the last medium change. Additions of 1.0 ml of 0.9% NaCl, with or without appropriate agonists or antagonists, were made to the 2.0-ml complete medium already on the plate; although such additions resulted in dilution of the original serum content, control plates did not show significant changes in the baseline levels of cAMP or in pH.

Measurement of cAMP. For the measurement of cAMP concentrations in intact cells, medium was removed rapidly and an aqueous acetic acid solution (1:1, v/v) was added as originally described (7). The resulting suspensions were heated for 3 min at 90° and centrifuged to remove cellular debris. Aliquots of the supernatant fluid were dried at 80° in small tubes and assayed for cAMP by the modified binding assay of Gilman (8, 9).

Cell density. Cell density was defined as the total amount of protein determined in the acetic acid suspension obtained from the cells scraped off a 60-mm Falcon plate, divided by the area of the plate. Protein was determined by the method of Lowry *et al.* (10). Such plates were not washed prior to the addition of acetic acid. Corrections for residual serum were made from a curve constructed by correlating protein values obtained in control experiments, in which plates were washed three times successively in 5.0 ml of 0.9% NaCl, with protein values obtained when plates were not washed. Protein values given are corrected and expressed as micrograms per square centimeter.

Determination of V_{max}, K_m, and K_b. Except where noted, *l*-epinephrine bitartrate, in solution either alone or with *dl*-propranolol hydrochloride, was applied for 2 min. Although whole cells were stimulated maximally in 5 min by epinephrine, a 2-min exposure was chosen to minimize leakage of cAMP into the medium or induction of phosphodiesterase activity (11). K_b[2] was determined in the following man-

[1] The abbreviation used is: cAMP, adenosine cyclic 3′,5′-monophosphate.

[2] K_b (12) is defined as the dissociation constant of a competitive antagonist and its receptor, determined by

$$\log\left[\frac{A'}{A} - 1\right] = \log(B) - \log K_b$$

where A'/A represents the dose ratio of agonist in the presence (A') and absence (A) of competitive antagonist giving equivalent response; B is the con-

ner. (a) A reference dose-response curve was determined for a given subculture of cells by exposing plates of cells to concentrations of epinephrine from 0.01 to 10 μM in the manner previously described (each order of magnitude divided into three divisions, two plates per division, duplicate assays of cAMP concentration per plate). (b) Two sets of plates from the same subculture were simultaneously exposed for 2 min (except where noted) to concentrations of propranolol from 0.001 to 10 μM and either 1 or 10 μM epinephrine. When variability was so great that construction of an accurate dose-response curve was not possible, the data were rejected totally. The effective dose of epinephrine for a response measured in the presence of propranolol (A') was then determined by reference to the dose-response curve. In contrast to the traditional method of determining K_b for propranolol (12), that is, a more limited range of propranolol concentrations and a wider range of epinephrine concentrations, the modifications used here allowed exploration of a wider range of cell density. For one experiment, 60–80 plates were used to determine a single K_b value; titration with propranolol in the presence of two fixed concentrations of epinephrine provided several points of overlap and gave a sufficient range of propranolol concentrations to establish whether inhibition was competitive. Both V_{max} and K_m values for epinephrine were determined by reference to the dose-response curve of a particular subculture. In all figures shown here, the smooth curves were arbitrarily drawn by hand to minimize the distance of the points to the curve.

Adenylate cyclase determinations. Cells were lysed in 20 mM glycylglycine buffer (pH 7.6)–5 mM $MgSO_4$. Aliquots of the cell lysate were incubated for 20 min at 30° with ATP in the presence of $MgSO_4$, theophylline, phosphoenolpyruvate, pyruvate kinase, Tris-HCl buffer (pH 7.6) (concentrations specified under RESULTS), and the appropriate agents in a shaker bath. Final volume was 200 μl. The reaction was terminated by boiling for 2 min (13). Under these conditions, formation of cAMP was found to be linear for 30 min. Precipitates were centrifuged, and 25 μl of the supernatant were assayed directly for cAMP, using the assay described above. V_{max} for epinephrine was determined in these preparations by incubating the homogenates with 10 μM epinephrine.

Phosphodiesterase activity.[3] Cyclic nucleotide phosphodiesterase activities were determined by a two-step method previously reported (14). Cells were homogenized in 40 mM Tris-HCl buffer (pH 8.0). A 50-μl aliquot was added to a reaction mixture (volume, 250 μl; composed of the homogenizing buffer with 3.75 mM 2-mercaptoethanol, 10 mM $MgCl_2$, and the indicated substrate concentrations of tritiated cAMP) and incubated in a shaker bath at 30° for 10 min. The mixture was boiled for 1 min, 25 μg of *Ophiophagus hannah* venom were added, and the tubes were incubated for 10 min at 30°; the reaction was then stopped by the addition of Bio-Rad AG 1-X8 resin. An aliquot of this mixture was removed for counting by liquid scintillation spectrometry.

RESULTS

Cell density and degree of cell-to-cell contact. Three general gradations of cell density were designated as follows. (a) no or minimal cell contact occurring within 2 days following subculture, characterized by a majority of cells isolated from one another (as determined by phase microscopy) and expressing morphological features characteristic of glial cells (10–24 μg of protein per square centimeter). A source of variability in this range of cell density may be explained by differences in the metabolic state of the parent cultures immediately prior to subculture. (b) Sparse to dense cells, characterized by a population in which all cells were in contact with other cells, but no cells had yet lost their distinct glial cell appearance (25–39 μg of protein per square centimeter). (c) Fully

centration of antagonist; and K_b, the dissociation constant, is read as the intercept in the plot of log $[A'/A - 1]$ vs. log (B).

[3] Phosphodiesterase determinations were carried out by Dr. C. Duttagupta of this laboratory.

confluent cells, in which all cells appeared to be rounded and to have lost most morphological characteristics of glial cells (40–100 μg of protein per square centimeter).

Variation of V_{max}, K_m, and K_b with cell density. These studies (Fig. 1) encompassed cultures used over 18 months, during which time, on several occasions, cultures were started from frozen pellets stored at −90°, so that a degree of variation with different batches of cell cultures was expected. When the same subculture was followed as it increased in cell density, the increase in V_{max} was quite evident (uninterrupted line in Fig. 1). In the range of the most active proliferation (15–35 μg of protein per square centimeter), doubling of cell density appeared to result in significantly more than a 2-fold increase in V_{max}.

In view of the possible sources of variation in the cultures used for experimentation, it was thought that a more distinct positive correlation with cell density could be found when the ratio of V_{max} to basal cAMP levels in the whole cell (Fig. 2) was determined as a function of cell density. There indeed appears to be an enhancement of the correlation, despite the fact that basal levels of cAMP may represent or include contributions from as yet undescribed catecholamine-insensitive cyclases that need not exhibit the same sensitivity

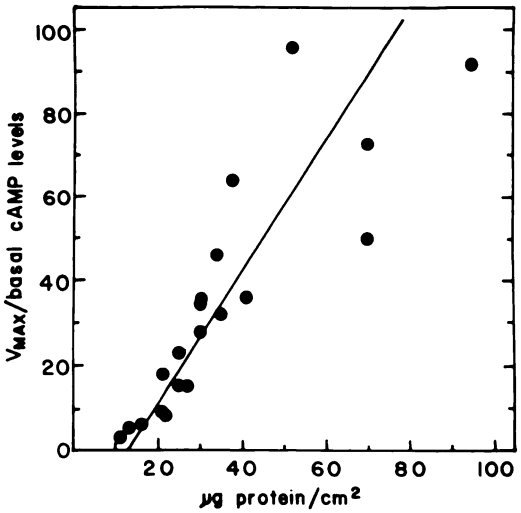

FIG. 2. *Correlation of ratio of V_{max} to baseline cAMP accumulation as a function of cell density*

Baseline cAMP levels are those accumulated in 2 min in the presence of serum when cells were present in the same volume of 0.9% NaCl but without hormone added. The line represents linear regression analysis of the data (correlation coefficient, 0.86; $p << 0.01$, with a slope of 0.65).

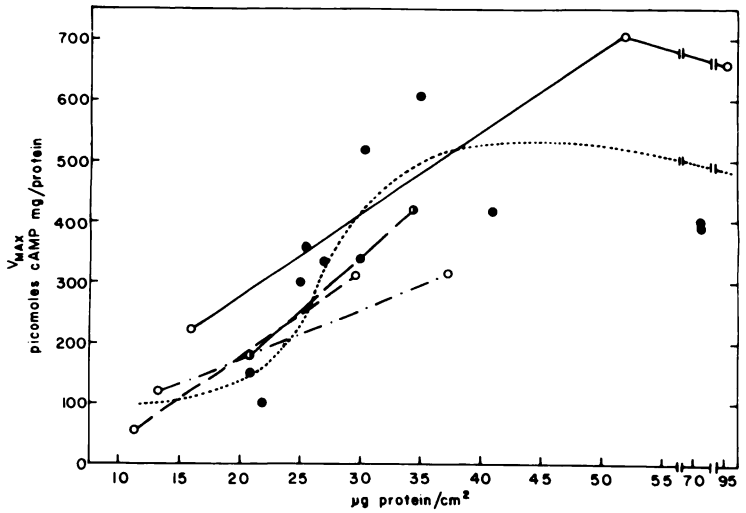

FIG. 1. *cAMP formation in intact C-6 astrocytoma cells: relationship to cell density and maximal response to epinephrine*

V_{max} (maximal activity in response to saturating concentrations of epinephrine in the presence of serum, as determined by reference to the dose-response curve of the particular subculture as described in the text) is expressed as total picomoles of cAMP accumulated per milligram of protein in 2 min. ○, the same subculture followed over time; ●, individual assays.

to cell density changes (13). The general pattern of the baseline levels of cAMP in the whole cell is, first, an increase with increasing cell density, and then a gradual decrease to virtually undetectable levels.

As shown in Fig. 3, individual subcultures (represented by uninterrupted lines) showed a distinct correlation of the K_m for epinephrine with increasing cell density. Statistical analysis (Table 1) of the three density gradations clearly indicated this positive correlation with cell density. The greatest rise in K_m appeared to be associated with the most active period of cell proliferation (15–35 μg of protein per square centimeter). In contrast, when serum was absent from the medium during assay, the K_m for epinephrine remained constant and, accordingly, independent of cell density (Table 2).

The correlation of cell density with K_b values for propranolol was more striking than with the K_m for epinephrine or with V_{max} values. Again, the greatest increase in K_b values occurred when the cells were proliferating most actively. However, when the cells had been incubated with propranolol for 45 min prior to a 2-min exposure to epinephrine, the K_b was reduced to a value of 1–2 nM, which apparently was independent of cell density (○——○, Fig. 4). Prior incubation with propranolol had

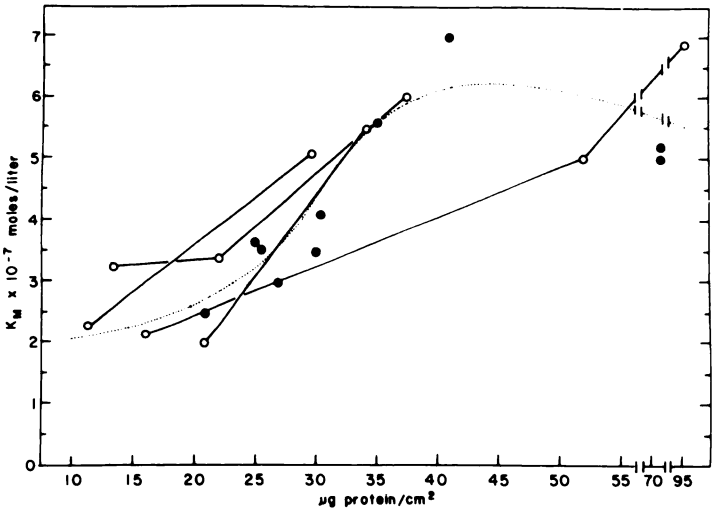

FIG. 3. *Correlation of K_m (concentration of epinephrine at which half-maximal stimulation of adenylate cyclase occurs in the presence of serum) with cell density*

○——○, values obtained by following the same subculture over time; ●, individual assays.

TABLE 1

Statistical analysis of composite data of Figs. 1–4 when cast into three gradations of cell density as described in results

Values are the means and standard deviations of the number of determinations given in parentheses.

Cell density	V_{max}	K_m	K_b	V_{max}/basal cAMP
μg protein/cm²	*pmoles cAMP/mg*	*nM*	*nM*	
10–24	141 ± 57 (7)	260 ± 56 (6)	2.43 ± 0.51 (6)	7.0 ± 5.66 (7)
25–39	390 ± 107[a] (9)	446 ± 165[a] (6)	6.78 ± 1.34[a] (6)	34.2 ± 14[a] (9)
40–100	515 ± 154[a, b] (5)	582 ± 100[a, c] (6)	9.76 ± 1.5[a, d] (6)	69.36 ± 20[a, c] (5)

[a] $p < 0.005$ with respect to the cell density of 10–24 μg/cm².
[b] $p < 0.10$ with respect to the cell density of 25–39 μg/cm².
[c] $p < 0.025$ with respect to the cell density of 25–39 μg/cm².
[d] $p < 0.05$ with respect to the cell density of 25–39 μg/cm².
[e] $p < 0.005$ with respect to the cell density of 25–39 μg/cm².

TABLE 2

cAMP formation in intact C-6 astrocytoma cells: influence of omission of serum on V_{max} and K_m for epinephrine and K_b for propranolol

Plates were washed once in Dulbecco's phosphate-buffered NaCl, allowed to equilibrate for 2 hr in the same medium, and assayed as previously described. Preliminary incubation in propranolol was carried out for 45 min as described.

Cell density	V_{max}	K_m	K_b	K_b after preliminary incubation
μg protein/cm²	*pmoles cAMP/mg*	*nM*	*nM*	*nM*
17.32	435	110	1.2	0.5
53.64	535	330	7.6	1.2
55.7	700	95		
94	930	100	10	1.4

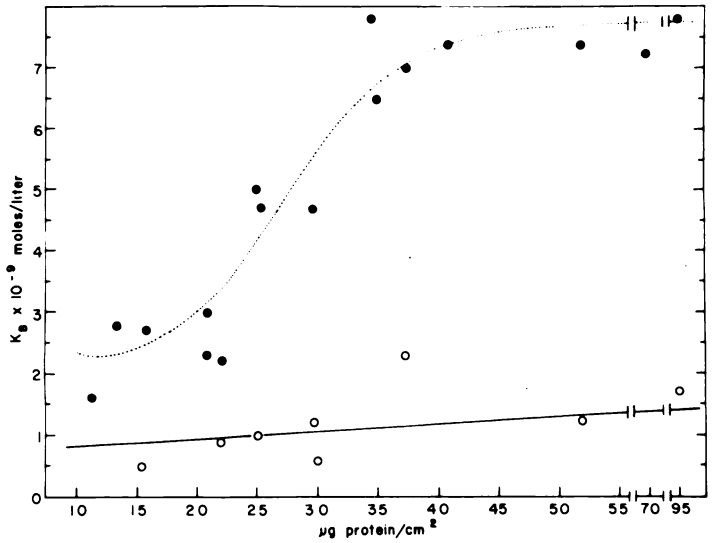

FIG. 4. *Correlation of K_b for propranolol with cell density, determined in the presence of serum*

○——○, values obtained after incubation of cells with propranolol for 45 min prior to the 2-min exposure to epinephrine; ●····●, values obtained after simultaneous incubation of propranolol and epinephrine for 2 min.

no effect on the activity of either the fluoride-stimulated adenylate cyclase or phosphodiesterase.[4]

The K_b values obtained by simultaneous incubation of propranolol and epinephrine and the K_m values for epinephrine exhibited a linear correlation with one another (Fig. 5). This relationship depended on the presence of serum when the K_m was determined, and on simultaneous incubation with propranolol and epinephrine for 2 min when K_b was measured. Thus the absence of serum from the incubation reduced the K_m to a nearly constant value (Table 2), whereas previous incubation with propranolol reduced the K_b to a nearly constant value independent of cell density (Fig. 4).

Role of serum. Because previous investigators had explored related cell density phenomena only in the absence of serum, a study was done on the time course of effects in glial tumor cells with and without serum.

The data in Fig. 6 represent the qualitative response to changing from a medium with serum to one without serum of a particular subculture of glial cells followed over time. From this, and from other experiments not shown, the basal concentra-

[4] Table 4 and unpublished observations.

tion of cAMP appeared to be more sensitive to a change of medium when cells were at low density. Thus cells at low but not high density showed an apparent initial increase and then a final decrease to a new steady-state level that was only slightly higher (at most 2-fold) than those levels occurring in the presence of serum (Fig. 6a). These changes occurred within 2 hr. Nontransformed 3T3 cells, when changed to medium without serum, attain after 20 hr a higher final steady-state baseline that is significantly more than twice the baseline of the control plates assayed in the presence of serum (15).

Following a change to medium with no serum, the response to maximum concentrations of epinephrine also first increased and then decreased to a value that, at 2 hr, was higher than the corresponding value observed in the presence of serum (Fig. 6b), and again these changes appeared to be more marked for lower- than for higher-density cell cultures.

Homogeneity of cell dispersions. As shown in Table 3, if the cell cultures on the plates were not homogeneously dispersed, the previously obtained relationships between cell density and the V_{max} and K_m for epinephrine and K_b for propranolol were no longer observed. This result confirms the hypothesis that these relationships are indeed due to cell density and not merely the total mass of cells per plate. We cannot yet explain the tremendously high values obtained for the heterogeneous preparations at the low average densities. However, they must represent primarily the contributions of the highly dense patches of cells, which were in the majority. The remainder of the plate was occupied by cells, relatively few in number, that showed minimal contact. It should also be noted that the cells in dense patches showed almost complete loss of the morphological character of glial cells, whereas the sparse patches retained the usual morphology. As the average cell density increased in such poorly dispersed preparations, the over-all appearance became essentially like that of cell cultures prepared with good dispersion and grown to high density.

Trypsinization. To rule out any contribution to this phenomenon of the tryspsinization procedure prior to subculturing, the maximal response of whole cells to saturating concentrations of epinephrine

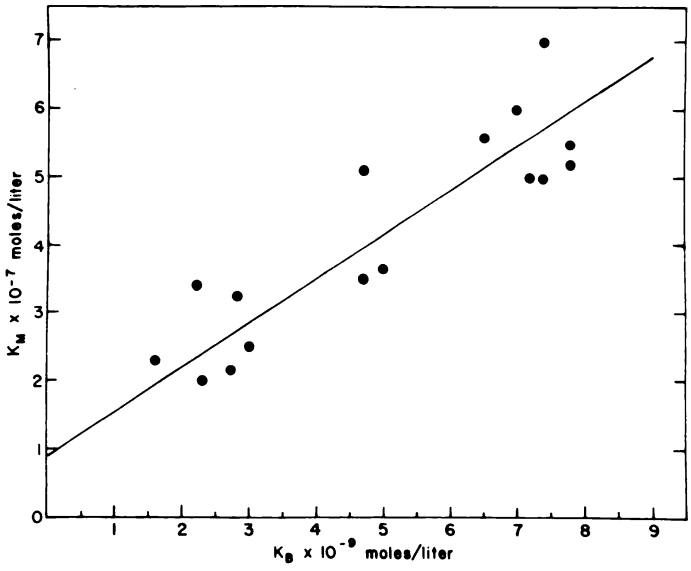

FIG. 5. *Correlation of K_m with K_b*

The line represents linear regression analysis fit, with a slope of 0.72. Data were derived from Figs. 3 and 4.

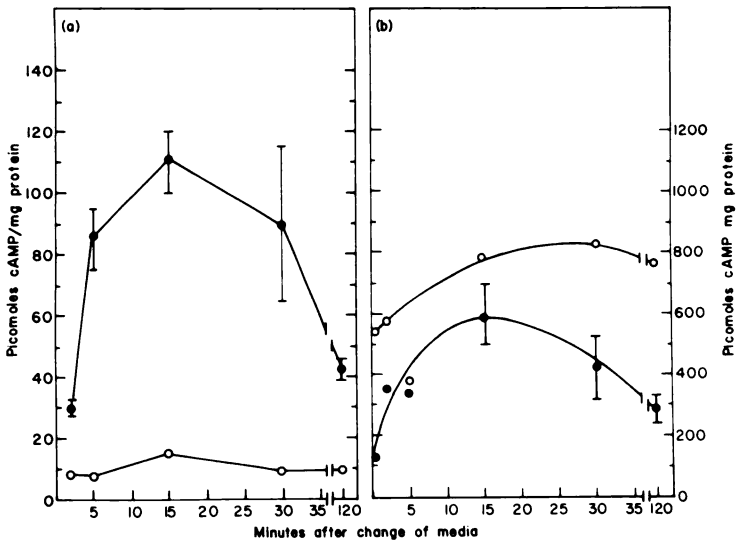

FIG. 6. *cAMP formation in intact cells: effect of changing medium with 10% serum to medium without serum on baseline measurements and maximally stimulated epinephrine activity*

(a) Cells of the same subculture, grown to the densities indicated (●, 19 μg/cm²; ○, 78 μg/cm²) in full Dulbecco's medium, were changed to Dulbecco's medium without serum and incubated at 37° for the times noted. Response to maximal concentrations of epinephrine (b) were assayed in the same manner as those shown in Fig. 6a, except that epinephrine (added in saturating concentration, 10 μM) was added during the last 2.0 min of incubation. Points at zero time in Fig. 6b represent activities at 2 min determined by reference to Fig. 1, i.e., in the presence of serum.

TABLE 3

Influence of nonhomogeneity of cell culture on determination of V_{max} and K_m for epinephrine and K_b for propranolol in intact cells

In these experiments cells were analyzed as before, but during subculture they were seeded without prior vortex-dispersion, resulting in points of highly dense (clustered) and nondense (minimal) growth on the same plate. Hence cell density represents the average protein on a series of plates, and is not at all indicative of the degree of contact.

Average cell density	K_m	K_b	V_{max}
μg protein/cm²	*nM*	*nM*	*pmoles cAMP/2 min*
31	2200	98	2800
43	620	22.5	1900
53	390	17.5	875
73	370	13.3	750

was measured in preparations 2 days after seeding at low and high cell concentrations. The ratio of V_{max} to basal cAMP levels in the low-density plates (13.5 μg/cm²) was 2.8, whereas the same ratio in the high-density plates (50 μg/cm²) was 62, values expected from Fig. 2. Hence the relationship of V_{max} to cell density is due solely to cell density and not to the interval between trypsinization and subculturing.

Adenylate cyclase and phosphodiesterase activities in relation to cell density. Studies were carried out to ascertain the relationship of the whole-cell phenomenon to its subcellular components in broken-cell preparations. The results shown in Fig. 7 indicate clearly that the baseline enzymatic activities and the maximal activities which resulted from stimulation by either epinephrine or NaF increased in relation to increasing cell density. Fluoride-stimulated adenylate cyclase activity was used as an index of cyclase activation that occurs via a mechanism different from hormonal stimulation (16).

Studies on cAMP phosphodiesterase activity (Table 4) revealed that, as cell density increased, the activity of the low-K_m enzyme for cAMP also increased. In contrast, even with tripling of cell density, no

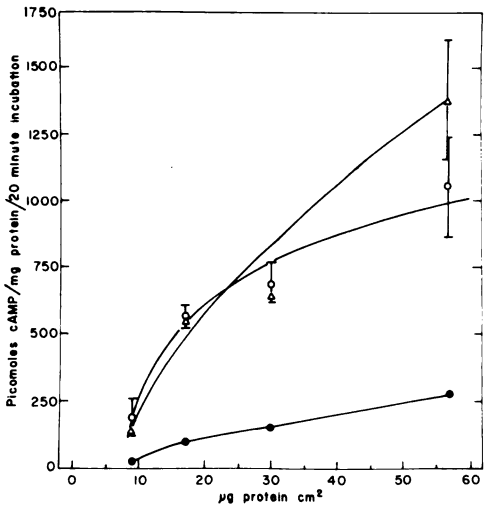

FIG. 7. *Adenylate cyclase activity and cell density*

Cells were washed three times in Dulbecco's phosphate-buffered NaCl (pH 7.4), scraped into 1.0 ml of the same buffer, and centrifuged at 500 rpm for 10 min at 4°, and the pellet was homogenized in various volumes of 5 mM $MgSO_4$–20 mM glycylglycine buffer (pH 7.4); 50 μl of the enzyme mixture (containing 50–80 μg of protein) were added to 150 μl of reaction mixture (over ice) containing final concentrations as follows: 40 mM Tris-HCl (pH 7.6), 5 mM theophylline, 2.5 mM $MgSO_4$, and 1.125 mM ATP. An ATP-regenerating system, as described in MATERIALS AND METHODS, and the various stimulatory agents were also present. Reactions were carried out as described in the text. Standard deviations are negligible (average ±10%) for baseline values. ●, basal activity; △, epinephrine (10 μM)-stimulated activity; ○, fluoride-stimulated activity.

significant change in the activity of high-K_m cAMP phosphodiesterase could be detected.

DISCUSSION

To our knowledge, this report on the rat astrocytoma C-6 glial cell is the first detailed study of the variation with cell density of properties of both the epinephrine receptor for adenylate cyclase (as determined by the K_b for propranolol) and the epinephrine receptor-mediated activation of adenylate cyclase (K_m for epinephrine and V_{max}) in the intact cell. It also describes how these relationships are affected by the presence and absence of serum. Furthermore, these cell density studies have been extended to assays with broken-cell preparations to permit analysis of individual components (adenylate cyclase and phosphodiesterase) that are large contributors to the events measured in the whole cell.

TABLE 4

Influence of cell density on phosphodiesterase activity

Activity was determined as described in MATERIALS AND METHODS. Activity of the enzyme with low K_m was determined in the presence of 5 μM cAMP, and activity of the enzyme with high K_m was determined in the presence of 100 μM cAMP. Preliminary incubation in propranolol was carried out as described in Table 2.

Conditions	Cell density	Phosphodiesterase activity	
		Low-K_m enzyme (5 μM)	High-K_m enzyme (100 μM)
	μg protein/cm²	*pmoles adenosine formed/mg protein/min*	
Control	9.5	41.22 (2)	680 (2)
Propranolol	9.5	36.3 (3)	531 (4)
Control	25	200 (2)	503 (2)

The results here confirm an earlier report (2) that the V_{max} of the catecholamine-sensitive adenylate cyclase of the whole cell increases with increasing cell density in the absence of serum. In contrast, work (3) using the human cloned cerebral glioblastoma tumor multiforme 1181N1 describes a relationship opposite to that found here with respect to cell density and V_{max} in the whole cell. Although the rat C-6 and the human cerebral glioblastoma lines are both transformed glial cells, at least one property, the capacity to synthesize increased amounts of S100 protein with increased cell density, is found in the rat C-6 line and not the human line (17). Species differences may extend to adenylate cyclase activity as well. In this work with human glioblastoma tumor multiforme 1181N1 (3), incubation with the hormone was performed immediately after the medium with serum was changed to medium without serum, and for 5 min thereafter. The response of the cells to this change of medium may have masked the significant response to the catecholamine

stimulus (see Fig. 6). A similar explanation may be offered for the differing results reported for the nontransformed line, WI-38 (4), obtained in a manner similar to that for the human glioblastoma tumor multiforme 1181N1 (3). In the experiments reported, in agreement with our present results (1, 2, 13), final exposure to the catecholamine was preceded by a lengthy incubation period in the medium without serum to which the cells had been changed. However, the relationship between V_{max} and cell density may be unique to the individual cell line studied, and have no special connection with serum.

The results in the present studies agree with the findings in WI-38 cells (4) that, in the absence of serum, there is no significant difference in K_m with different cell densities. This supports the contention that an increase in cell density is not accompanied by a change in the sensitivity of the catecholamine receptor–adenylate cyclase complex. Furthermore, in other experiments with C-6 cells (data not presented here), we found that the K_m for epinephrine does not change significantly when velocities are measured at 5 rather than 2 min. Even at the higher cell densities, diffusion for epinephrine does not appear to be rate-limiting.

In the present report, the presence of serum appears responsible for the elevation of the apparent K_m for epinephrine with increasing cell density. Serum has previously been reported to suppress the maximum response to epinephrine of cells in culture (1); the possible relationship of this effect on V_{max} to the effect on K_m reported here is not known. The possibility that serum might enhance the destruction of epinephrine, and that this loss of agonist may be more critical at the higher cell densities, has not been evaluated.

When propranolol and epinephrine were added simultaneously for 2 min, the K_b determined at the higher cell densities was markedly increased. This suggests that cell-to-cell contact decreases the intrinsic affinity of the receptor (12). The finding that incubation with propranolol for 45 min prior to exposure to epinephrine gave a constant K_b independent of cell density implies that the ability of propranolol to establish rapid equilibrium with the receptor is restricted at the higher cell densities; studies using homogenates of heart have shown that blocking by propranolol is not complete before 2 min (18), and studies with homogenates of Ehrlich ascites tumor cells did not rule out a 2-min lag time (19). However, a slow onset of action by propranolol is insufficient to explain the elevation of K_b with increasing cell density, since the assays were performed under identical conditions at both high and low densities. Furthermore, there appears to be no diffusion barrier for epinephrine at higher cell densities, and under conditions in which the K_m remains constant independent of cell density (assays in the absence of serum) the K_b remains elevated at higher cell densities. In preliminary experiments we have found that millimolar concentrations of unlabeled propranolol only partially displace tracer-tritiated propranolol bound to these intact cells. Thus a large reservoir of nonspecific propranolol binding sites may exist in these cells. Furthermore, experiments not reported here indicate that incubation of whole cells with propranolol, followed by washing and homogenization, results in persistent blockade of the epinephrine-stimulatable adenylate cyclase, presumably by re-equilibration of this trapped propranolol with the reaction mixture and subsequent blockade of the specific receptor, as suggested by results similar to these (20, 21). An augmented concentration of these nonspecific binding sites at higher cell densities could reduce the effective concentration of propranolol at the specific receptors, resulting in the apparent elevation of K_b with increasing cell density. If the nonspecific binding sites have a lower affinity but faster rate of association, the preliminary incubation period would be required to establish equilibration. Further studies will be needed to confirm this possibility.

Despite a difference in the method of determining K_b values, the value reported here for the dissociation constant K_b of propranolol (1–2 nM obtained after preliminary incubation) agrees well with values reported for kitten right atria, 3 nM (18),

and for turkey erythrocyte ghosts, 2.5 nM, that were obtained by direct measurement of binding of ^{3}H-labeled propranolol and by competition with adenylate cyclase activation (22).

That these correlations of V_{max}, K_m, and K_b require homogeneous cell-to-cell contact and are not simply artifacts of the trypsinization procedure is indicated both by the results obtained with the cultures dispersed nonhomogeneously (Table 3) and by the ratios of V_{max} to basal cAMP levels measured in plates 2 days after seeding at low and high cell concentrations. Thus neither total cell mass nor time after trypsinization can account for the phenomenon.

When these density-related studies were extended to measurements with broken-cell preparations, the basal and fluoride- and epinephrine-stimulatable adenylate cyclase activities were found to increase with cell density, as did phosphodiesterase activity, data in agreement with other reports (1, 13, 23, 24). Such an increase in phosphodiesterase activity does not negate the augmented capacity of intact cells to respond to epinephrine at higher cell densities but might be responsible for the lack of increase in basal levels of cAMP measured in these preparations. In conclusion, the total activity (V_{max}) of the epinephrine-sensitive adenylate cyclase in the whole cell of the rat astrocytoma C-6 line increases with increasing cell density without a change in the apparent affinity of the receptor or the sensitivity of the receptor-cyclase complex. Furthermore, the subcellular components of the whole-cell phenomenon all increase with increasing cell density. On the basis of these data, one may consider that cell-to-cell contact is associated with either (a) the appearance *de novo* of additional receptor-cyclase units, or the activation and incorporation of nascent units into the membrane, thus increasing the concentration of such subunits per milligram of protein, or (b) an increase in the specific activity of the adenylate cyclase linked to the receptor.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Mrs. Elaine Keehn. We are indebted to Dr. S. Seifter and Mr. D. L. Prinstein for reviewing the manuscript.

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