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8-Bromo-3′,5′-Adenosine Monophosphate Stimulates the Endocrine Activity of Human Cytotrophoblasts in Culture*

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ABSTRACT. Cytotrophoblasts, purified from human term placentae, were cultured in the absence or presence of 8-bromo-cAMP or 8-bromo-cGMP. 8-Bromo-cAMP provoked a dose-dependent increase in the secretion of hCG and progesterone within 24 h. After 48 h, hCG secretion increased by more than 200-fold, and progesterone secretion increased nearly 5-fold. 8-Bromo-cGMP had no effect on hCG secretion. In culture in serum-supplemented medium, the mononuclear cytotrophoblasts aggregated and fused to form syncytia. This morphological transformation was not affected by 8-bromo-cAMP. Immunocytochemical studies of the α- and β-subunits of hCG in control and 8-bromo-cAMP-stimulated cultures demonstrated that the cyclic nucleotide analog promoted the synthesis of both subunits in all cellular forms, including single mononuclear cells, cell aggregates, and syncytia. In serum-free medium, the cytotrophoblasts did not aggregate or form syncytia, yet they responded to 8-bromo-cAMP with an increase in hCG secretion. We conclude that the endocrine function of cytotrophoblasts can be stimulated by a cAMP-dependent mechanism which can be initiated independently of the formation of a syncytium. (*J Clin Endocrinol Metab* **63**: 1211, 1986)

THE SYNCYTIOTROPHOBLAST of the human placenta expresses several endocrine functions, including secretion of protein and steroid hormones (1). It is thought that the syncytiotrophoblast is formed by fusion of mononuclear cytotrophoblasts, which are the mitotically active component of the trophoblast (2–4). Syncytiotrophoblasts react strongly when histological sections are stained immunocytochemically for hCG and human placental lactogen, whereas the cytotrophoblasts do not stain (5). These findings suggest that the mononuclear cytotrophoblasts are incapable of elaborating these protein hormones. The transformation of cytotrophoblasts into syncytiotrophoblasts has been difficult to study *in vitro* because of the lack of suitable cell preparations and culture systems. Recently, we described the isolation of purified cytotrophoblasts from human term placentae (5). We found that cytotrophoblasts aggregated in culture and then fused to form syncytia during a 72-h period (5). Although the freshly isolated cells did not stain for hCG, after 48 h of culture, the cells spontaneously started to secrete appreciable quantities of the hormone. These observations indicate that cytotrophoblasts undergo both morphological and functional differentiation in culture.

Some endocrine functions of the trophoblast can be stimulated by cAMP analogs. These analogs stimulate hCG secretion by placental tissue (6–10) and trophoblast and nontrophoblast neoplasms (6, 7, 10–12). Caritis *et al.* (13) reported modest stimulation of progesterone secretion by trophoblast cells in culture in response to $(Bu)_2$cAMP. Similar findings were reported by Tonkowicz and Poisner (14). However, in the previous studies on normal placental tissue, it is not clear whether syncytiotrophoblasts and/or cytotrophoblasts were responding to the cAMP analogs.

We were interested in knowing if cyclic nucleotides influence the expression of endocrine activities of purified cytotrophoblasts, and whether these second messengers affect the morphological transformation of cytotrophoblasts into syncytia. Here we report that 8-bromo-cAMP, but not 8-bromo-cGMP, accelerates the onset of hCG secretion and augments its production as well as the secretion of progesterone. In contrast, 8-bromo-cAMP does not alter the rate at which the cells aggregate

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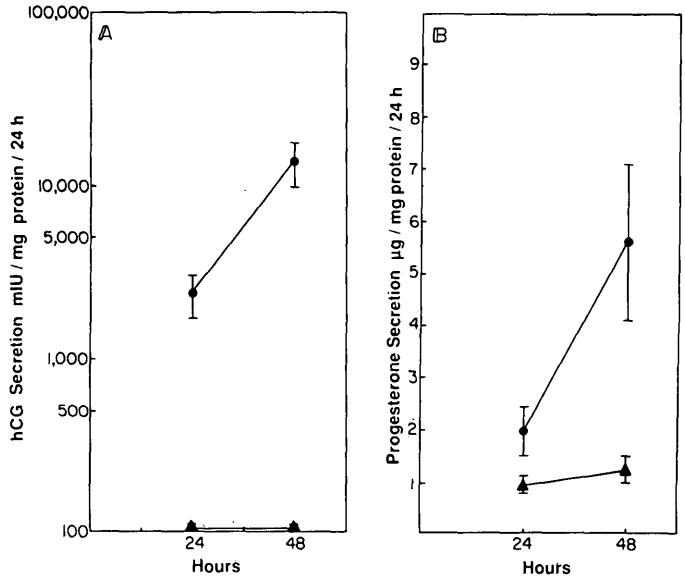

FIG. 1. Effects of 8-bromo-cAMP on hCG and progesterone secretion by cultured cytotrophoblasts. Cells were cultured for 48 h in the absence (▲) or presence (●) of 8-bromo-cAMP (1.5 mM). hCG (A) and progesterone (B) were quantitated in the medium at 24-h intervals. Values presented are the mean ± SE from six separate experiments. At each time point, 8-bromo-cAMP-treated cultures secreted significantly more ($P < 0.014$, by the Wilcoxon signed rank test) progesterone and hCG than did control cultures.

and fuse to form syncytia. Thus, trophoblast endocrine activity is independent of syncytia formation.

Materials and Methods

Preparation and culture of cytotrophoblasts

Cytotrophoblasts were isolated from term placentae obtained immediately after spontaneous vaginal delivery or uncomplicated cesarean section, as described by Kliman *et al.* (5). Briefly, after trypsin-DNase digestion of 30 g soft villous tissue, as described by Hall *et al.* (15), the cell suspensions were pooled and placed on a Percoll gradient. The gradient was made from 5–70% Percoll (vol/vol) in 5% steps of 3 ml each by dilutions of 90% Percoll (9 parts Percoll to 1 part 10X Hanks' medium) with calcium and magnesium-free Hanks' medium. The gradient was centrifuged at 1200 × *g* at room temperature for 20 min. The middle band (density, 1.048–1.062 g/ml) containing the cytotrophoblasts was removed, washed once with Dulbecco's modified Eagle's Medium containing 25 mM HEPES and 25 mM glucose (DMEM-H-G), and then resuspended in medium for tissue culture. More than 95% of the isolated cells were cytotrophoblasts, as assessed by immunocytochemistry. The cytoplasm of the isolated cells stained positively with a polyclonal antibody to a 34K growth factor which is localized to cytotrophoblasts of intact placenta (16), but did not stain for the β-subunit of hCG. Based on the immunocytochemical detection of markers for macrophages (α_1-antichymotrypsin) and endothelial cells and fibroblasts (vimentin), we estimated that less than 5% of the cells in the preparation were contaminants (5). Cell viability, as assessed by trypan blue exclusion, was 95% or greater.

Cytotrophoblasts, diluted to a concentration of 4 or 8 × 10^5 cells/ml (for 16- or 35-mm dishes, respectively) with DMEM-H-G containing 4 mM glutamine and 50 µg/ml gentamicin with or without 20% (vol/vol) heat-inactivated fetal calf serum were plated in 16- or 35-mm Nunclon culture dishes (Nunc, Roskilde, Denmark) and incubated in humidified 5% CO_2-95% air at 37 C. Some 35-mm dishes contained a 22-mm square glass coverslip (no. 1). In some experiments, cyclic nucleotide analogs were added to the medium at the time of plating, while in others, 8-bromo-cAMP was added to cultures after incubation for 48 h.

At various times, culture media were changed, and coverslips were removed for immunocytochemical staining. At the termination of biochemical studies, cells were scraped from the dishes with a rubber policeman. Protein contents of cells, solubilized in 0.2% Triton X-100 in 1 N NaOH, were measured by the method of Bradford (17), using BSA as a standard.

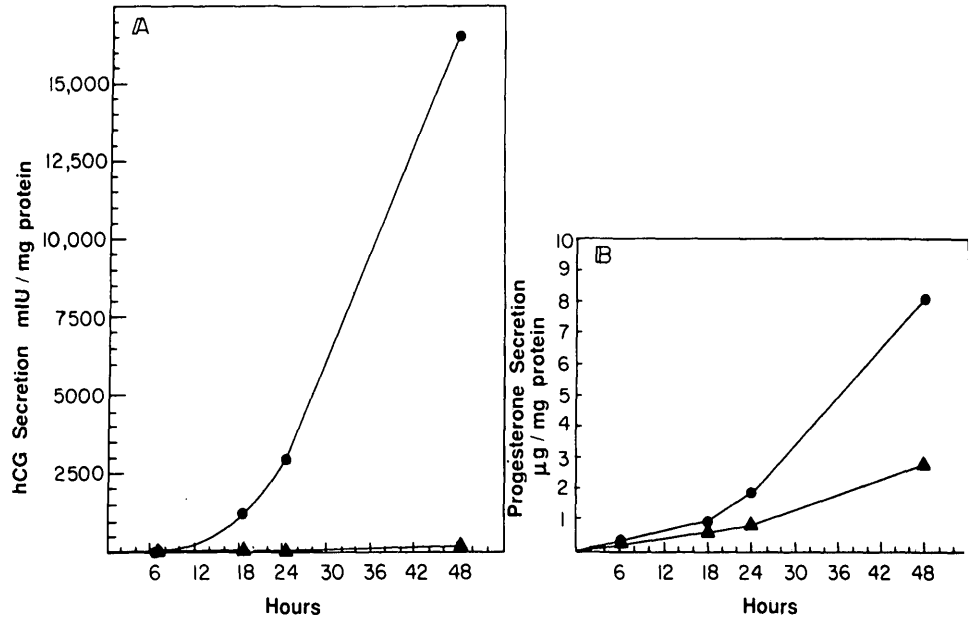

FIG. 2. Time course of 8-bromo-cAMP effects on hCG and progesterone secretion by cultured cytotrophoblasts. Cytotrophoblasts were cultured in the absence (▲) or presence (●) of 8-bromo-cAMP (1.5 mM). At the indicated time periods, media were collected for assay of hCG (A) and progesterone (B). The values shown are the mean cumulative hCG and progesterone secretion from triplicate cultures. Hormone levels in the replicate cultures varied less than 10%.

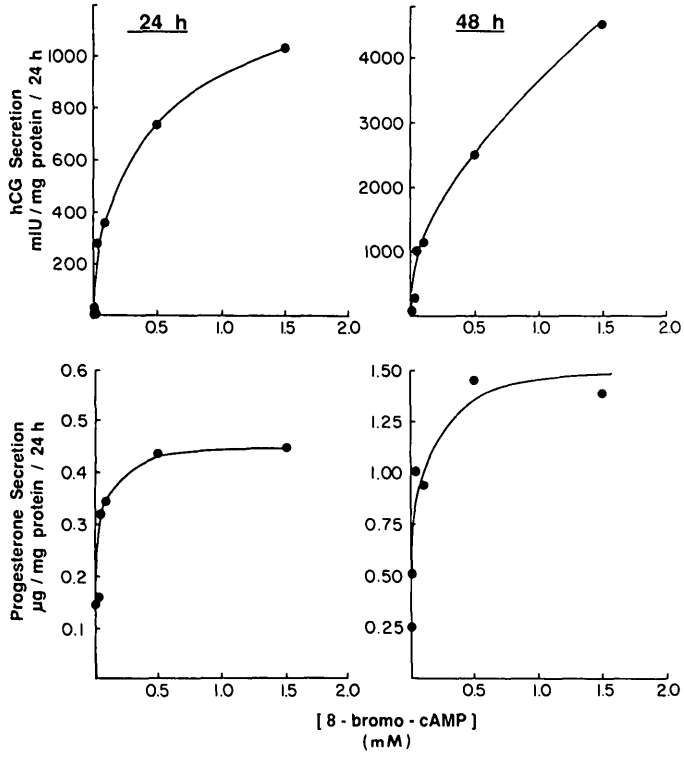

FIG. 3. Effects of various concentrations of 8-bromo-cAMP on hCG and progesterone secretion by cultured cytotrophoblasts. Cytotrophoblasts were cultured in the absence or presence of the indicated concentrations of 8-bromo-cAMP for 48 h. Media were changed at 24-h intervals. hCG and progesterone were measured by RIA. Values presented are means from triplicate cultures in which replicate values varied by less than 10%.

TABLE 1. Effects of 8-bromo-cAMP and 8-bromo-cGMP on hCG secretion by cultured cytotrophoblasts

Treatment	hCG secretion (mIU/mg protein·24 h)	
	24 h	48 h
Control	38 ± 7[a]	32 ± 6[a]
8-Bromo-cAMP	1504 ± 407[b]	8312 ± 2183[b]
8-Bromo-cGMP	64 ± 27[a]	245 ± 144[a]

Cytotrophoblasts were cultured in the absence or presence of 8-bromo-cAMP (1.5 mM) or 8-bromo-cGMP (1.5 mM) for a 48-h period. Media were changed at 24-h intervals, and hCG was quantitated by RIA. Values presented are the mean ± SE from four separate experiments. For each time period, means with different superscripts are significantly different ($P < 0.01$) by the Mann-Whitney test.

Immunocytochemical staining of cultured trophoblasts

Coverslips were washed twice with 150 mM NaCl-10 mM phosphate buffer, pH 7.4 (PBS), fixed for 15 min with Bouin's solution, and washed twice with PBS at room temperature. Coverslips were stored in PBS at 4 C before staining. All subsequent steps were performed at 24 C. The coverslips were washed twice with PBS and once with PBS-1% BSA (PBS-BSA). Endogenous peroxidase activity was quenched by a 15-min incubation with 0.6% H_2O_2 in PBS. Nonspecific immunoglobulin G-binding sites were blocked by a 30-min incubation with 5% goat serum (Dako, Santa Barbara, CA) in PBS. Before the next and all subsequent reactions, the coverslips were washed twice with PBS and once with PBS-BSA. Primary antisera raised in rabbits were diluted in PBS and applied to the coverslips for 45 min. These included antibodies against the β-subunit of hCG (Dako) at a 1:1000 dilution and the α-subunit of hCG (gift from Drs. Canfield and Birken, Columbia Medical Center, New York, NY) at a 1:800 dilution. The primary antibodies were visualized using an avidin-biotin-peroxidase detection method (18) with a kit purchased from Vector Laboratories (Burlingame, CA). 3,3′-Diaminobenzidine was used as the color reagent; it produces a dark brown deposit. After the reaction with 3,3′-diaminobenzidine, the coverslips were rinsed twice with tap water, counterstained with hematoxylin, dehydrated in absolute ethanol, dipped in xylene until cleared, and mounted on glass slides with Preservaslide (E. M. Science, Cherry Hill, NJ).

Assay of hCG and progesterone

hCG was quantitated by RIA using reagents purchased from Corning Medical (Medfield, MA). The antibody was specific for the β-subunit, and the assay had a sensitivity of 1.5 mIU (Second WHO International Standard)/ml. The results are expressed as milliinternational units standardized to the Second WHO International Standard. Progesterone was quantitated using a specific RIA, as previously reported (19).

The culture media contained no detectable hCG or progesterone. Addition of aminoglutethimide, an inhibitor of cholesterol side-chain cleavage, to the medium (50 µg/ml) completely inhibited progesterone secretion by the cultured cells.

Statistical analysis

Values are presented as the mean ± SE of the indicated number of separate experiments, each performed with a different cytotrophoblast preparation. The Mann-Whitney test or Wilcoxon signed rank test was used to determine significant differences between treatment groups (20).

Results

Purified cytotrophoblasts cultured in serum-supplemented medium with 8-bromo-cAMP (1.5 mM), added at the time of plating, resulted in a marked stimulation of hCG secretion (Fig. 1A). The control cells secreted less than 50 mIU hCG/mg protein·24 h during the initial 48 h of culture, whereas the 8-bromo-cAMP-treated cultures secreted 3,500 mIU/mg protein·24 h during the initial day of culture and more than 10,000 mIU/mg protein·24 during the subsequent day. The secretion of progesterone by the cells was also enhanced by 8-bromo-cAMP (Fig. 1B). Progesterone secretion was increased almost 2-fold over control levels during the first 24 h of culture and almost 5-fold during the subsequent 24 h.

The response of the cultured trophoblasts to 8-bromo-cAMP was evident within 18 h (Fig. 2) and was dose dependent (Fig. 3). The progesterone response was max-

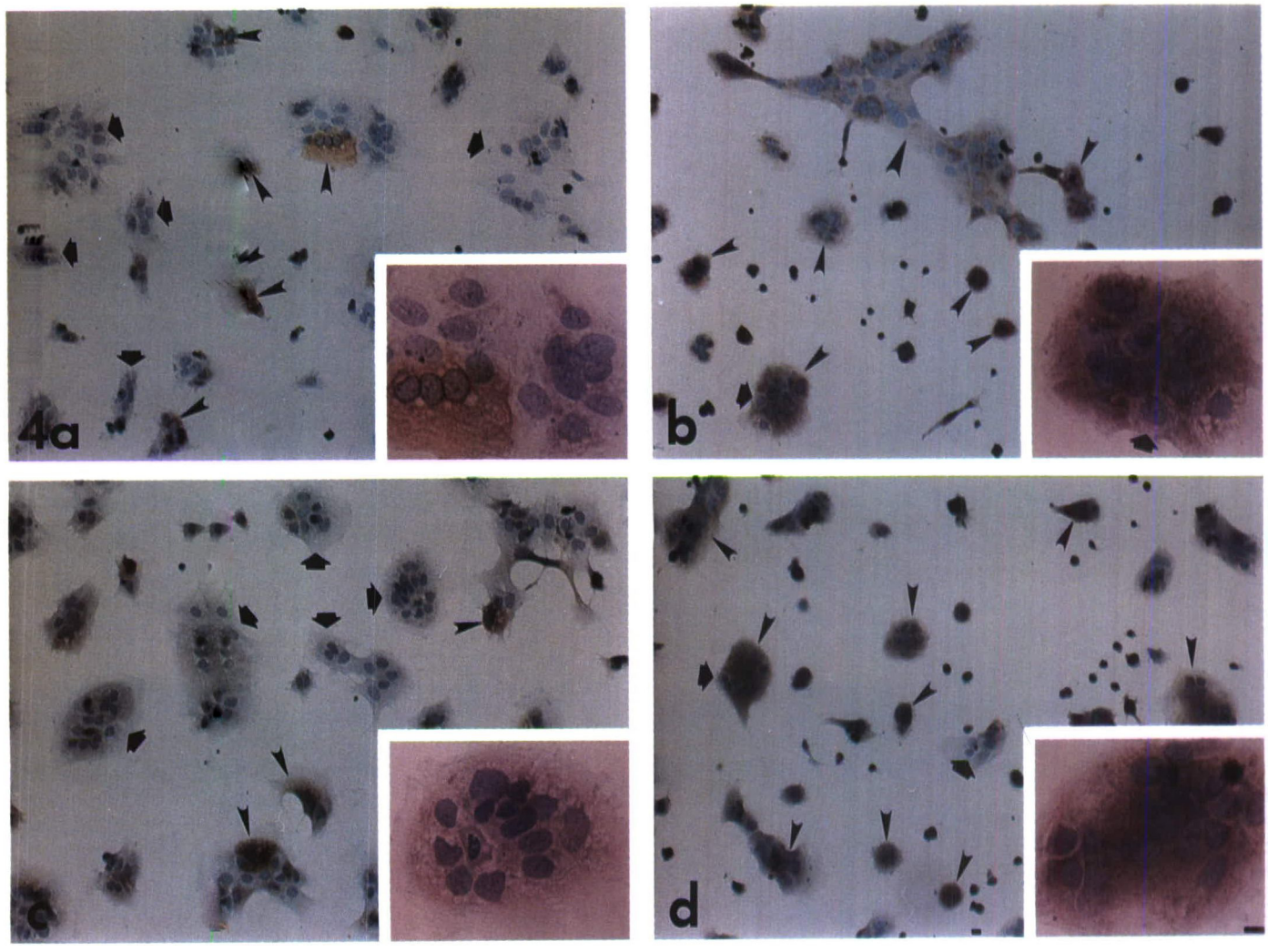

FIG. 4. Effect of 8-bromo-cAMP on aquisition of the α- and β-subunits of hCG in cultured trophoblasts. Cytotrophoblasts were cultured for 48 h without (a and c) or with (b and d) 1.5 mM 8-bromo-cAMP and immunocytochemically stained for αhCG (a and b) or βhCG (c and d), as described in *Materials and Methods*. a) Scattered trophoblasts stain for αhCG (*arrowheads*) in the absence of 8-bromo-cAMP, but the majority of the cells are negative (*arrows*). *Inset*, Mixed aggregate and syncytium showing immunopositivity in the syncytium while the remaining cells are negative. b) Virtually all trophoblasts are positive for αhCG in the presence of 8-bromo-cAMP (*arrowheads*). A large mixed aggregate-syncytium exhibits patchy cytoplasmic positivity (*large arrowhead*). *Inset*, Aggregate with diffuse positivity. Note single negative cell (*arrow*). c and d) Analogous results using antibodies against βhCG without and with 8-bromo-cAMP. All main micrographs are at the same magnification, and all insets are at the same magnification. *Bars in lower right corner* of d represent 10 μm.

imal with 0.5 mM 8-bromo-cAMP, while the hCG response was increasing even at 1.5 mM. Unlike 8-bromo-cAMP, 8-bromo-cGMP (1.5 mM) did not significantly increase hCG secretion by the cultured cells (Table 1).

8-Bromo-cAMP also had a profound effect on the immunocytochemical staining of the cultured trophoblasts as early as 18 h (Figs. 4 and 5). At 18 h, less than 10% of the trophoblasts in the aggregate stage contained α- or βhCG immunoreactivity in the absence of 8-bromo-cAMP, while more than 90% of these cells stained when treated with 8-bromo-cAMP. Single cells also showed an increase in immunostaining in the presence of 8-bromo-cAMP. At 18 h, the few syncytia that were present were immunopositive for α- or βhCG, even in the absence of 8-bromo-cAMP; thus, a differential increase in immunostaining was not detected. At 48 h, more single cells and aggregated cells were positive for α- or βhCG in the absence of 8-bromo-cAMP, but, again, the addition of 8-bromo-cAMP greatly increased the immunostaining. The difference was most dramatic for cells in the aggregate stage of differentiation. As at 18 h, syncytia at 48 h were already largely positive, but 8-bromo-cAMP seems to have promoted a noticeable increase in immunostaining for βhCG (Fig. 5).

8-Bromo-cAMP did not grossly alter the progression of cultured trophoblasts to aggregates or syncytia (Fig. 5, *top panel*). However, a consistent change in cellular configuration was found in the presence of the cyclic nucleotide. As with malignant trophoblast cells (6) and human granulosa cells (21), the cAMP analog caused rounding up of the trophoblasts. This finding was most notable at 18 h of treatment and decreased with contin-

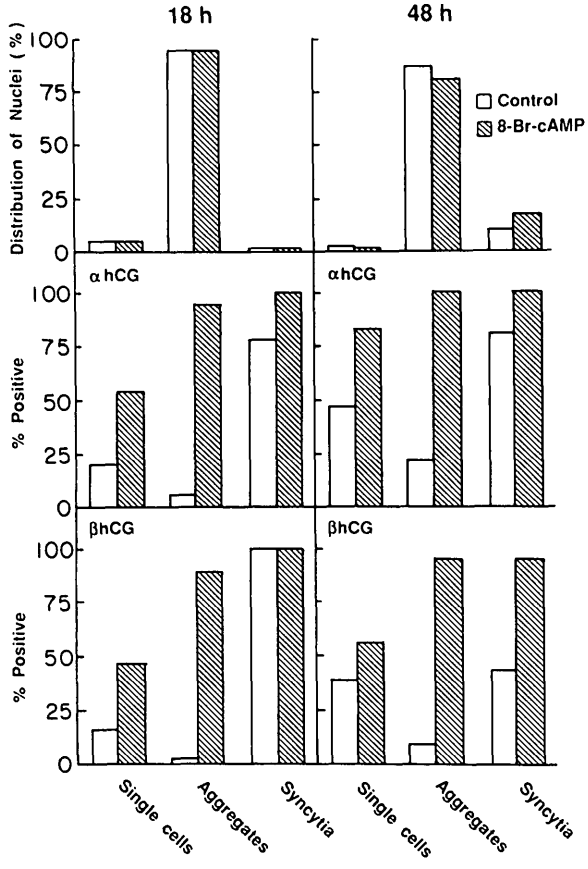

FIG. 5. Effect of 8-bromo-cAMP on acquisition of the α- and β-subunits of hCG by cultured trophoblasts. Cytotrophoblasts were cultured without (□) or with (▨) 1.5 mM 8-bromo-cAMP, as described in *Materials and Methods*. At 18 and 48 h, the cells were fixed and immunocytochemically stained to localize the α- or β-subunit of hCG. *Top panels*, Trophoblast morphology. Beginning at a random point near the middle of each coverslip, 500 nuclei were counted in sequential high power fields and assessed as to whether they were in single isolated cells, single cells of an aggregate (2 or more cells attached to each other or single cells attached to syncytia), or syncytia. *Middle panels*, Percent cellular staining for αhCG in the 3 trophoblast forms. *Lower panels*, Percent cellular staining for β-hCG in the 3 trophoblast forms. Any discernible staining was considered positive. In aggregates, only nuclei of stained cells were considered positive. All nuclei in a syncytia were considered positive if any part of the cytoplasm was stained.

ued exposure. In addition to the rounding up of the cultured trophoblasts, 8-bromo-cAMP induced a marked decrease in the number of cytoplasmic vacuoles (Fig. 6, c and d).

Addition of 8-bromo-cAMP to cultures established for 48 h in serum-supplemented medium also resulted in an increase in hCG and progesterone secretion during the subsequent 24 h of incubation (Table 2). Between 48 and 72 h of culture, the control cells spontaneously began to secrete hCG. However, the cyclic nucleotide analog still increased hCG production more than 7-fold. Progesterone secretion was also enhanced.

When the cytotrophoblasts were plated in serum-free medium, the cells remained spherical, with no evidence of flattening out onto the substratum even after 120 h in culture (Fig. 6). Although the cells did not form aggregates or syncytia, they still synthesized hCG in the presence of 1.5 mM 8-bromo-cAMP. Treatment with the cyclic nucleotide analog for 48 h resulted in the secretion of 945 and 3247 mIU hCG/mg protein·24 h during the first and second days of culture, respectively. Control cultures secreted 32 and 126 mIU hCG/mg protein·24 h. The same cytotrophoblasts cultured in serum-supplemented medium aggregated and began to form syncytia. In the control state, these cells secreted 18 and 23 mIU hCG/mg protein·24 h during the first and second days of culture, respectively, whereas cells stimulated with 8-bromo-cAMP secreted 598 and 2127 mIU hCG/mg protein·24 h, respectively, during the same time periods. Thus, cells cultured in serum-free medium had a biochemical response to the cyclic nucleotide equivalent to that of cells cultured in serum-supplemented medium. Moreover, immunocytochemical studies confirmed that 8-bromo-cAMP treatment provoked expression of hCG formation in the cells cultured in serum-free medium, whereas control cells did not stain for the hormone.

Discussion

Cytotrophoblasts are the progenitors of the syncytiotrophoblasts. Our previous (5) and present results demonstrate that cytotrophoblasts differentiate in serum-supplemented medium and secrete hormones characteristic of the syncytiotrophoblast. cAMP accelerates this functional differentiation independent of the formation of syncytia. Culturing cytotrophoblasts in serum-free medium provided a striking demonstration of this property. The cells failed to aggregate and fuse, but still increased their hCG secretion in response to 8-bromo-cAMP. Serum may contain factors required for syncytial formation. Adding serum to cells cultured for 2–3 days in serum-free medium resulted in aggregation and fusion of the single cells (our unpublished observations).

cAMP analogs are known to stimulate hCG secretion in placental explants, dispersions, and tumor cells (6–12). In culture of malignant trophoblast, these analogs increase the number of cells that stain immunocytochemically for hCG (22). Our study describes the action of 8-bromo-cAMP on a purified preparation of cytotrophoblasts. The magnitude of the hCG and progesterone responses to the cAMP analog was greater than previously described (8–10, 13, 14). This marked increase may be explained by the greater concentration of cytotrophoblasts in our system.

The response to cAMP analogs depends on the stage of functional differentiation of the cells. This was demonstrated by adding 8-bromo-cAMP to the cultures after 48 h. At this time, the cells had begun to aggregate and spontaneously secrete hCG. The stimulation of hCG secretion was less than that which occurred after adding

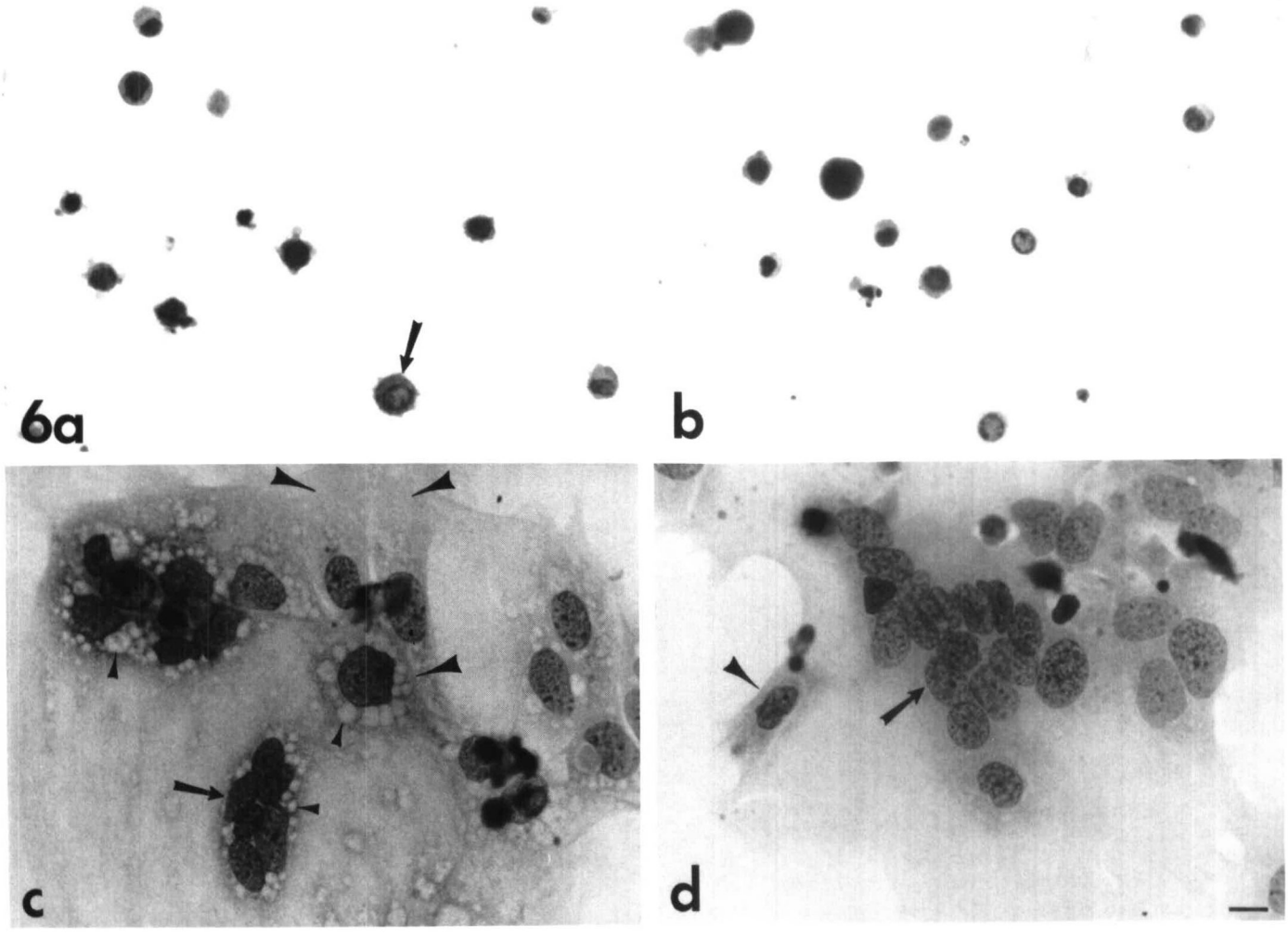

FIG. 6. Effects of fetal calf serum and 8-bromo-cAMP on cultured trophoblasts. Cytotrophoblasts were cultured in the absence (a and b) or presence (c and d) of 20% fetal calf serum without (a and c) or with (b and d) 1.5 mM 8-bromo-cAMP for 72 h, as described in *Materials and Methods*. a) Cytotrophoblasts remaining as individual cells. Most are sperhical and, therefore, dense, but occasional cells flattened out sufficiently to reveal some cytoplasmic detail (*arrow*). b) The 8-bromo-cAMP-treated cells appeared similar. c) In the presence of 20% fetal calf serum, the cytotrophoblasts flattened out, aggregated, and formed syncytia. Multiple nuclei can be seen in the center of a large syncytium (*arrow*). At the periphery of the syncytium, individual cells (*large arrowheads*) can be seen. Note the perinuclear vacuoles (*small arrowheads*). d) The 8-bromo-cAMP-treated cultures appeared similar, except for the loss of cytoplasmic vacuoles. Note multinucleated syncytia (*arrow*) and mononuclear trophoblast (*arrowhead*). All micrographs are at same magnification; the *bar* represents 10 μm.

TABLE 2. Effects of 8-bromo-cAMP on hCG and progesterone secretion by cytotrophoblasts established in culture for 48 h

Treatment	hCG secretion (mIU/mg protein·24 h)	Progesterone secretion (μg/mg protein·24 h)
Control	962 ± 288	1.62 ± 0.11
8-Bromo-cAMP (1.5 mM)	7499 ± 3124	2.97 ± 0.62

Cytotrophoblasts were cultured for 48 h, as described in the text. 8-Bromo-cAMP was added to some cultures, and incubations were continued for 24 h. hCG and progesterone were measured in the incubation medium. Values presented are the mean ± SE from three separate experiments. 8-Bromo-cAMP significantly increased hCG and progestin secretion ($P < 0.025$, by the Mann-Whitney test).

the cyclic nucleotide at the time of plating (7.8- *vs.* 40-fold).

The stimulatory effects we described were cAMP specific. This finding agrees with the known abundance of cAMP-dependent protein kinase compared to the cGMP-dependent kinase in the human placenta (23). In contrast to our results, Hilf and Merz (10) reported that 8-bromo-cGMP and 8-bromo-cAMP similarly increased hCG secretion by placental explants. These researchers found only a small increment in hCG secretion in response to the cyclic nucleotides. We cannot offer an explanation for the variance in the results. The specific response to cAMP is probably encoded in the 5′-flanking sequence of the appropriate genes (24). Presumably, cAMP increases the production of mRNAs from these same genes (25).

While our studies establish the capacity of cytotrophoblasts to respond to cAMP, we have not determined which hormone or paracrine or autocrine factor(s) might increase cellular cAMP levels *in situ*. There are several possible candidates, including catecholamines, which activate placental adenylate cyclase (26). Menon and Jaffe (27) reported that hCG stimulated placental adenylate cyclase. This finding raises the intriguing possibility of a paracrine/autocrine function for hCG with respect to differentiation of cytotrophoblast endocrine function. GnRH, which is formed by the placenta (28), stimulates hCG secretion by placental explants (8, 29, 30). Epidermal growth factor could also up-regulate endocrine activity in the trophoblast (31).

There are interesting similarities between the development of the syncytiotrophoblast and a corpus luteum. Mitotically active cytotrophoblasts eventually undergo a process of functional differentiation. Stimulated by a cAMP-dependent mechanism, they acquire the synthetic potential of the mature syncytiotrophoblast. Similarly, follicular granulosa cells, which divide during follicular growth, undergo a cAMP-dependent process of luteinization that augments their endocrine capacities (32). Both the syncytiotrophoblast and luteal cell secrete large quantities of progesterone, and both also secrete protein hormones. We suggest that there are common molecular mechanisms at work in the functional differentiation of these two cell types.

Acknowledgments

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