

# Cyclic Adenosine-3',5'-monophosphate Stimulates Islet B Cell Replication in Neonatal Rat Pancreatic Monolayer Cultures

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**ABSTRACT** A possible role for cyclic adenosine-3',5'-monophosphate (cAMP) in islet B cell replication was examined in neonatal rat pancreatic monolayer cultures. Islet cells deteriorated and insulin release decreased during 12 d of culture in medium with 5.6 mM glucose, whereas the cells survived and insulin release increased during culture in medium with 5.6 mM glucose plus the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 0.1 mM), or in medium with 16.7 mM glucose with or without IBMX. IBMX also increased the mitotic index and stimulated dose-dependent increases in [<sup>3</sup>H]thymidine incorporation in nuclei of islet B cells in aldehyde-thionine stained radioautographs; maximal stimulation of B cell replication occurred with addition of 0.1 mM IBMX to 5.6 mM glucose (+170%,  $P < 0.001$ ), and this increase was similar to that observed with 16.7 mM glucose (+185%,  $P < 0.001$ ). Also, 8-bromo-adenosine-3',5'-monophosphate, but not 8-bromo-guanosine-3',5'-monophosphate produced dose-dependent increases in islet B cell replication in medium with 5.6 mM glucose. Measurement of cAMP levels in the cultures revealed dissociations between effects on B cell replication and insulin release. Thus, addition of 0.1 mM IBMX, or 0.1 nM cholera toxin, to 5.6 mM glucose produced slightly greater increases in cAMP levels and B cell replication than did 16.7 mM glucose, whereas insulin release was increased significantly more with 16.7 mM glucose. Also, addition of 0.1 mM IBMX, or

0.1 nM cholera toxin, to 16.7 mM glucose stimulated further increases in cAMP levels and insulin release in the cultures, but no further increases in B cell replication. We conclude that (a) cAMP stimulates islet B cell replication, (b) cAMP may mediate the effects of glucose on B cell replication, and (c) mechanisms regulating B cell replication may be more sensitive to cAMP and/or different from those regulating insulin secretion.

## INTRODUCTION

Development of tissue culture methods for the study of pancreatic islet B cell function has demonstrated that glucose occupies a central role in the regulation of a variety of B cell functions. Thus, culture of pancreatic islets, or islet cells, in the presence of high concentrations of glucose (15–20 mM) has been shown to increase glucose metabolism (1, 2), insulin release and content (3–6), and insulin biosynthesis (7), and also to improve the survival (6) and to stimulate the replication (8–11) of islet B cells. Glucose has also been reported to stimulate adenylate cyclase (5, 12) and to increase cyclic adenosine-3',5'-monophosphate (cAMP) levels (13) in cultured islets. In view of recent reports that cAMP stimulates proliferation in a variety of epithelial cells (14–16), the present study was undertaken to determine whether this cyclic nucleotide might also stimulate replication in pancreatic islet B cells, and, thereby, might play a role in glucose-induced B cell replication. Accordingly, neonatal rat pancreatic monolayer cultures were maintained in the presence of agents known to increase cAMP levels in islets (3-isobutyl-1-methyl xanthine [IBMX],<sup>1</sup> 8-bromo-adenosine-3',5'-monophosphate; IBMX, 3-isobutyl-1-methylxanthine; PBS, phosphate-buffered saline.

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<sup>1</sup>Abbreviations used in this paper: 8-Br-cAMP, 8-bromo-adenosine-3',5'-monophosphate; IBMX, 3-isobutyl-1-methylxanthine; PBS, phosphate-buffered saline.

sine-3',5'-monophosphate [8-Br-cAMP], cholera toxin), and the time- and dose-dependent effects of these agents on islet B cell replication and insulin levels in the cultures were measured.

## METHODS

**Preparation of islet cell monolayer cultures.** A modification of the method of Lambert et al. (17) was used. 3-d-old Wistar-Lewis inbred rats (Charles River Breeding Laboratories, Wilmington, Mass.) were killed by decapitation. The pancreases were removed aseptically in a laminar flow hood, and placed in cold (4°C) sterile phosphate-buffered saline (PBS), pH 7.0. The pancreatic tissue was washed three times in calcium- and magnesium-free PBS, finely minced for 5 min, washed with gentle magnetic stirring for 10 min, then dissociated at 37°C by adding 10 ml per 100 pancreases of freshly prepared calcium- and magnesium-free PBS (pH 7.4) containing 5.6 mM glucose, 5 mg/ml of trypsin (Difco Laboratories, Detroit, Mich., 1:250), and 0.2 mg/ml of collagenase (CLS IV, Worthington Biochemical Corp., Freehold, N. J.). After 8 min gentle magnetic stirring at 37°C, the tissue was allowed to settle and the supernatant fluid was decanted. Fresh trypsin-collagenase solution was added to the residual tissue and the digestion and decantation procedure was repeated ~10 times, until the tissue was dissociated into single cells and small clusters. The first two supernates were discarded and subsequent supernates were pooled and diluted with cold (4°C) culture medium 199 with modified Earle's salts, L-glutamine, and phenol red (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.), buffered with 25 mM sodium bicarbonate and supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), fungizone (0.25 µg/ml), 10% fetal calf serum, and 16.7 mM glucose (standard culture medium). The cells were centrifuged for 10 min at 300 g. The pellet of cells was then resuspended in fresh medium.

To establish monolayer cultures, the cells were diluted with standard culture medium to  $\sim 7.5 \times 10^5$  cells/ml, transferred into 100-mm plastic culture dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.), and incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Fibroblasts attached to the bottom of the dishes more rapidly than did islet cells, so that after 15 h of incubation, the primary cell suspension was decanted into new dishes, thereby increasing the ratio of islet to fibroblastic cells in the cultures. A second decantation into 35-mm dishes, 5 h later, eliminated more fibroblasts. 44 h later, the standard culture medium was replaced by medium free of both cystine (18) and serum, for 24–36 h, until the great majority of fibroblasts were killed and detached. Standard culture medium was then replaced and, 4 d later, the islet cells had spread out in monolayer clusters.

Test and control media additions to the cultures were begun at this time (7 d after first plating the enzymatically-dissociated pancreatic tissue) and this was referred to as culture day zero. IBMX (Aldrich Chemical Co., Milwaukee, Wisc.), 8-Br-cAMP and 8-Br-cGMP (Sigma Chemical Co., St. Louis, Mo.), and cholera toxin (Schwartz/Mann Div. Becton, Dickinson & Co., Orangeburg, N. Y.) were dissolved in medium 199 containing 5.6 mM or 16.7 mM glucose. The media were sterilized by filtration through a membrane (0.45 µm, Millipore Corp., Bedford, Mass.) and added to the monolayer cultures (2 ml/35-mm culture dish).

**Insulin in media and cultured cells.** Media were collected from the cultures and replaced by fresh test or control media each 2–3 d, and after 2–12 d, the monolayers were extracted in acid-ethanol (19). Samples of culture media and acid-ethanol extracts of cultured cells were diluted in glycine buf-

fer (pH 8.6) containing 1 mg/ml of human serum albumin and assayed for insulin content by a charcoal separation method of radioimmunoassay (20). Purified porcine insulin was iodinated with <sup>125</sup>I by the chloramine-T method (21), and purified on a cellulose column (5 × 0.5 cm) by eluting with 20 mM iodoacetamide in normal plasma. Guinea pig anti-porcine insulin antiserum was used as antibody, and rat insulin was used as standard in the assay.

**Islet B cell replication studies.** During the last 18 h of a 2–12 d culture, the monolayers were incubated with fresh test or control media containing [*methyl*-<sup>3</sup>H]thymidine (10 µCi/ml, 40–60 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). The cultures were then washed three times with control medium to remove and dilute [<sup>3</sup>H]thymidine not incorporated into DNA, and reincubated for 32 h in control medium containing 5.6 mM glucose in order to regranulate the B cells. The cells were fixed with Bouin's solution and stained with aldehyde-thionine (22), and radioautographs were prepared (23) with Ilford L-4 photographic emulsion (Polysciences, Inc., Warrington, Pa.) (23). At least 500–1,000 aldehyde-thionine stained B cells were counted in each tissue culture dish and the percentage of these B cells containing [<sup>3</sup>H]thymidine-labeled nuclei was noted.

To determine that the [<sup>3</sup>H]thymidine incorporation index of islet B cells represented DNA replication, *N*-desacetyl-*N*-methylcolchicine (colcemid, 1 µg/ml, Gibco Laboratories) was added to the monolayers for the last 18 h of the culture period, in the place of [<sup>3</sup>H]thymidine, in order to arrest cells in metaphase. The cells were then swollen in hypotonic solution (0.075 M KCl) to improve subsequent identification of mitotic figures (24), fixed, stained, and the percentage of B cells in metaphase was determined by counting at least 500–1,000 B cells in each culture.

**DNA assays.** The DNA content of islet cell monolayer cultures was measured by the fluorophotometric method of Kissane and Robins (25), as modified for pancreatic islets by Green and Taylor (26).

**cAMP assays.** After collection of media and washing three times with PBS, the cultures were extracted in 6% trichloroacetic acid and their cAMP content was measured by using a radioimmunoassay kit (New England Nuclear, Boston, Mass.), and employing an acetylation method (27) that detected as little as 0.01 pmol cAMP per assay tube.

## RESULTS

**Time-dependent effects of glucose and IBMX on islet B cell survival.** Insulin release decreased progressively in islet cell monolayers cultured in medium containing 5.6 mM glucose (Fig. 1). In contrast, insulin release rates increased in cultures maintained in 5.6 mM glucose and 0.1 mM IBMX or in 16.7 mM glucose, without or with 0.1 mM IBMX. The total insulin release during 12 d of culture in 5.6 mM glucose and 0.1 mM IBMX (5042 ± 841 ng/dish, mean ± SEM) was significantly greater than that observed during culture in 5.6 mM glucose alone (875 ± 138 ng/dish, *P* < 0.001), and not significantly less than the insulin released during culture in 16.7 mM glucose (6,087 ± 817 ng/dish); addition of 0.1 mM IBMX to 16.7 mM glucose increased total insulin release during the 12-d culture to 8,417 ± 720 ng/dish (*P* < 0.05, vs. 16.7 mM glucose).

Fig. 2 shows that the islet cell monolayer clusters cultured in medium containing 5.6 mM glucose retracted

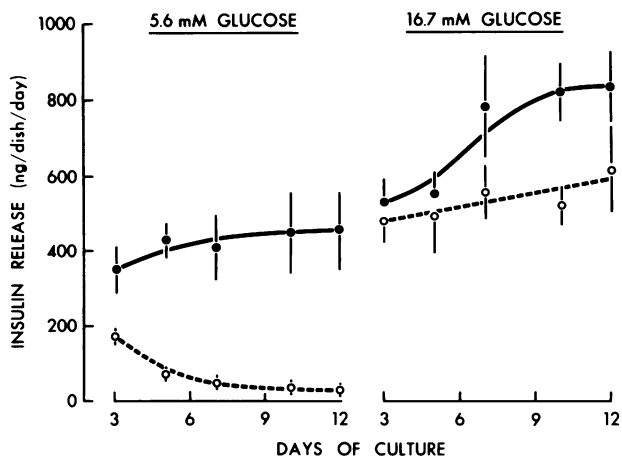


FIGURE 1 Time-course of the effects of glucose and IBMX on rates of insulin release in islet cell monolayer cultures. Media were collected from the cultures and replaced by fresh media each 2–3 d. Accumulation of insulin in medium, at the end of each collection period, is expressed as a daily rate (mean  $\pm$  SEM) for six culture dishes. (----), control; (—), +10<sup>-4</sup> IBMX.

and deteriorated during the 12-d culture, whereas islet cell monolayers cultured in 5.6 mM glucose and 0.1 mM IBMX, or in 16.7 mM glucose, without or with 0.1 mM IBMX, remained intact. The number of islet cells (DNA) recovered after 12 d of culture in 5.6 mM glucose was about one-half that recovered in cultures maintained in 5.6 mM glucose plus 0.1 mM IBMX, or in 16.7 mM glucose, without or with 0.1 mM IBMX (Table I). DNA recoveries in the cultures were accompanied by proportional changes in cellular insulin levels, suggesting that islet cell recovery (DNA) reflected islet B cell survival under the different culture conditions.

**Islet B cell replication studies.** Addition of 0.1 mM IBMX to 5.6 mM glucose increased [<sup>3</sup>H]thymidine incorporation in islet B cell nuclei by 88% ( $P < 0.01$ ) to a level not significantly different from that observed with 16.7 mM glucose, without or with 0.1 mM IBMX (Table II). Similarly, addition of 0.1 mM IBMX to 5.6 mM glucose increased the B cell mitotic index by 83% ( $P < 0.001$ ), and this was not significantly different from the effect observed with 16.7 mM glucose, without or with 0.1 mM IBMX.

The dose-dependent effects of IBMX on [<sup>3</sup>H]thymidine incorporation in islet B cells are shown in Fig. 3. In medium containing 5.6 mM glucose, increasing concentrations of IBMX (from 1  $\mu$ M to 0.1 mM) produced progressive increases in B cell labeling, whereas the highest concentration of IBMX tested (1 mM) was ineffective. Similarly, Fig. 4 shows that, in medium containing 5.6 mM glucose, islet cell survival was progressively improved by addition of IBMX, from 1  $\mu$ M to 0.1 mM, whereas 1 mM IBMX was toxic to the cells. Fig. 3 also shows that the stimulatory effect of 0.1 mM

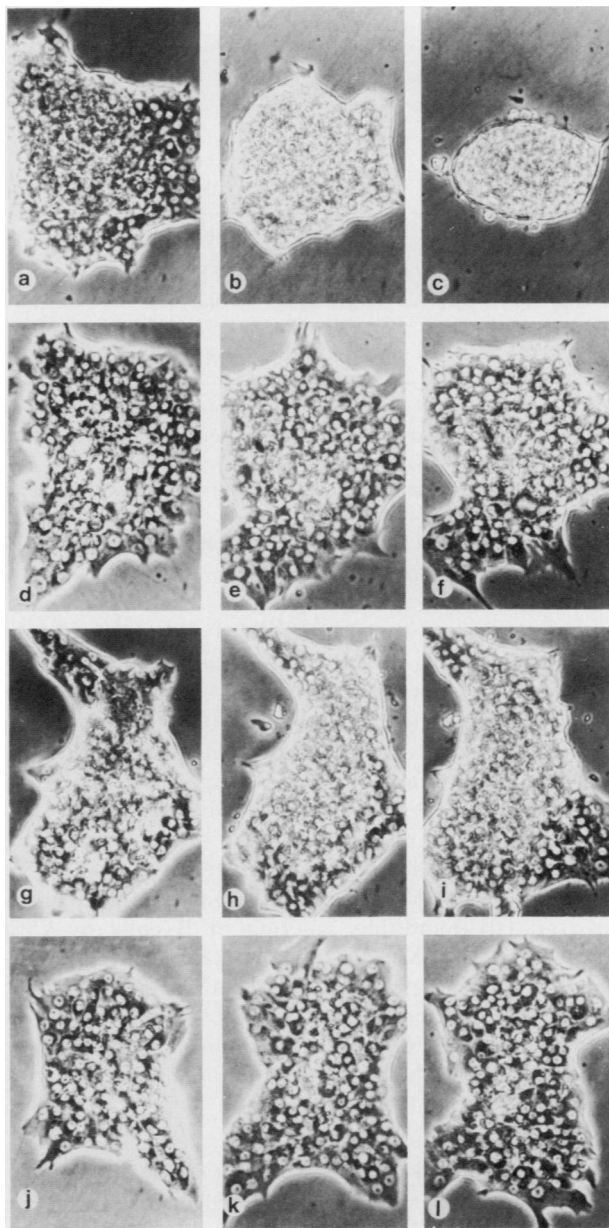


FIGURE 2 Phase-contrast photomicrographs ( $\times 400$ ) of pancreatic islet cells in monolayer culture in medium supplemented with 10% serum and glucose, with and without IBMX. A cluster of islet cells is shown on (a) day 0 (7 d after first plating the enzymatically-dissociated pancreatic tissue), (b) day 4, and (c) day 12 of culture in medium with 5.6 mM glucose. A second islet cell cluster is shown on (d) day 0, (e) day 4, and (f) day 12 in 5.6 mM glucose and 0.1 mM IBMX. A third islet cell cluster is shown on (g) day 0, (h) day 4, and (i) day 12 in 16.7 mM glucose. A fourth islet cell cluster is shown on (j) day 0, (k) day 4, and (l) day 12 in 16.7 mM glucose and 0.1 mM IBMX.

IBMX on B cell labeling in medium with 5.6 mM glucose (+170%,  $P < 0.001$ ) was similar to the effect of 16.7 mM glucose (+185%,  $P < 0.001$ ). Addition of in-

**TABLE I**  
Effects of Glucose and IBMX on Insulin and DNA Contents in Islet Monolayer Cultures

Additions to culture medium*		Insulin†	DNA‡	Insulin/DNA
Glucose	IBMX			
mM		ng/dish	ng/10 dishes	
5.6	0	64±5	88±12	0.73
5.6	0.1	142±17	193±15	0.74
16.7	0	132±22	164±5	0.80
16.7	0.1	158±30	189±29	0.84

\* Cultures were incubated for 12 d in media with additions as indicated, then all dishes were incubated in 5.6 mM glucose for 32 h to regranulate islet B cells.

† Acid-ethanol extractable insulin in the cultured cells; mean values±SEM for six dishes.

‡ Cells were pooled from 10 dishes for each DNA assay; mean values±SEM for three separate groups of 10 dishes.

creasing concentrations of IBMX (1 μM to 0.1 mM) to 16.7 mM glucose did not significantly change B cell labeling; however, addition of 1 mM IBMX to 16.7 mM glucose was clearly inhibitory ( $P < 0.001$ ), and this was accompanied by toxic effects to the islet cells (Fig. 4).

Because the phosphodiesterase inhibitor IBMX increases tissue levels of both cAMP and cGMP (28), these two cyclic nucleotides were tested, separately, for possible effects on islet B cell replication (Fig. 5). Addition of 10 μM 8-Br-cAMP to 5.6 mM glucose increased [<sup>3</sup>H]thymidine labeling of B cells by 75% ( $P < 0.05$ ), 0.1 mM 8-Br-cAMP increased B cell labeling by 133% ( $P < 0.01$ ), and 1 mM 8-Br-cAMP increased B cell labeling by 205% ( $P < 0.001$ ). Addition of 8-Br-cAMP to 16.7 mM glucose did not significantly increase B cell labeling above the level observed with

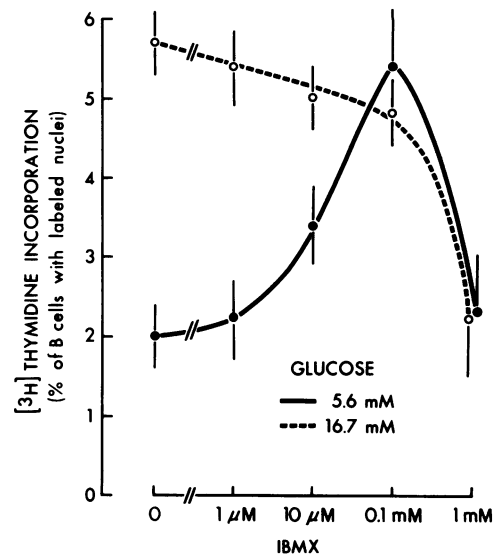
**TABLE II**  
Effects of Glucose and IBMX on Islet B Cell Replication

Additions to culture medium*		[ <sup>3</sup> H]Thymidine incorporation†	Mitotic index‡
Glucose	IBMX		
mM		% labeled B cells	% B cells in mitosis
5.6	0	3.4±0.3	2.4±0.1
5.6	0.1	6.4±0.5	4.4±0.2
16.7	0	5.6±0.3	4.5±0.5
16.7	0.1	6.3±0.5	5.1±0.5

\* Cultures were incubated for 12 d in media with additions as indicated.

† Cultures were incubated in 10 μCi/ml [<sup>3</sup>H]thymidine during the last 18 h of a 12-d culture; mean values±SEM for five dishes.

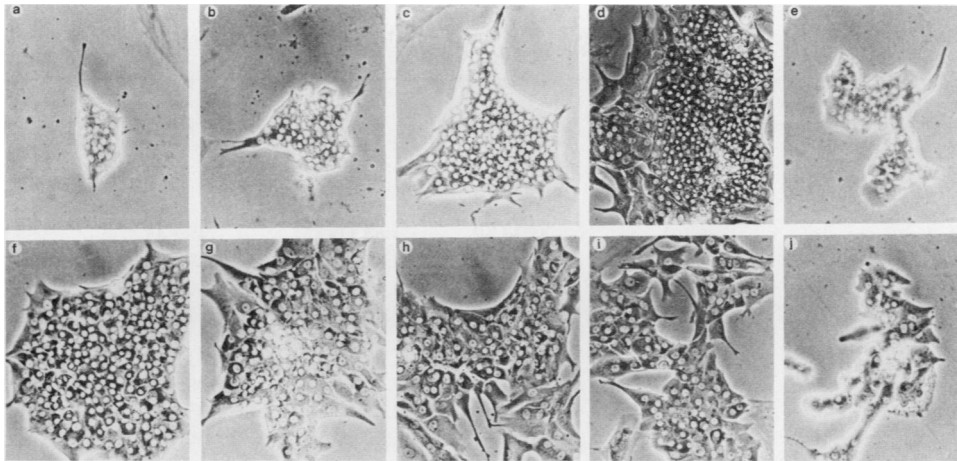
‡ Cultures were incubated in 1 μg/ml colcemid during the last 18 h of a 12-d culture; mean values±SEM for five dishes.



**FIGURE 3** Dose-dependent effects of IBMX (1 μM–1 mM) on [<sup>3</sup>H]thymidine incorporation in nuclei of islet B cells cultured for 12 d in medium containing 5.6 mM or 16.7 mM glucose. Each value is the mean±SEM for five culture dishes.

16.7 mM glucose. In contrast to the stimulatory effects of 8-Br-cAMP on [<sup>3</sup>H]thymidine labeling of islet B cells cultured in 5.6 mM glucose, 8-Br-cAMP (1 μM to 1 mM) had no significant effects in the presence of either 5.6 or 16.7 mM glucose.

**Comparison of the effects of cAMP on insulin release and B cell replication.** Cyclic AMP levels in islet cell monolayer cultures were increased by addition to the culture media of either the phosphodiesterase inhibitor, IBMX, or the adenylate cyclase stimulator, cholera toxin. The effects on insulin release and B cell replication were compared after 2 d of culture, at which time the number of cells present (DNA content of the cultures) was not significantly different for the different culture conditions (Table III). Addition of 0.1 mM IBMX to 5.6 mM glucose produced a slightly greater increase in cAMP (+118%,  $P < 0.05$ ) than did 16.7 mM glucose (+68%,  $P < 0.01$ ), and this was accompanied by a slightly greater increase in B cell replication with 5.6 mM glucose plus 0.1 mM IBMX (+214%,  $P < 0.001$ ) than with 16.7 mM glucose (+159%,  $P < 0.001$ ). In contrast, 16.7 mM glucose increased insulin release to a significantly higher level than that observed with 5.6 mM glucose plus 0.1 mM IBMX ( $P < 0.001$ ). Addition of 0.1 mM IBMX to 16.7 mM glucose stimulated further significant increases in cAMP levels and insulin release in the cultures, whereas B cell replication was not increased significantly above the level observed with 16.7 mM glucose alone. In another experiment, addition of 0.1 nM cholera toxin to 5.6 mM glucose produced greater increases in cAMP (+165%,  $P < 0.01$ ) and B cell replication (+166%,  $P < 0.001$ ) than did 16.7 mM glu-



**FIGURE 4** Phase-contrast photomicrographs ( $\times 400$ ) of pancreatic islet cells in monolayer culture after 12 d of incubation in medium containing 5.6 mM glucose: (a) alone, (b) + 1  $\mu$ M IBMX, (c) + 10  $\mu$ M IBMX, (d) + 0.1 mM IBMX, and (e) + 1 mM IBMX; and 16.7 mM glucose; (f) alone, (g) + 1  $\mu$ M IBMX, (h) + 10  $\mu$ M IBMX, (i) + 0.1 mM IBMX, and (j) + 1 mM IBMX.

ucose (+49%,  $P < 0.05$ ; and +110%,  $P < 0.001$ , respectively), whereas insulin release was increased to a significantly higher level with 16.7 mM glucose than with 5.6 mM glucose plus 0.1 nM cholera toxin ( $P < 0.001$ ). Addition of 0.1 nM cholera toxin to 16.7 mM glucose stimulated further increases in cAMP levels and insulin release in the cultures, whereas B cell replication was not significantly different from that observed with 16.7 mM glucose alone.

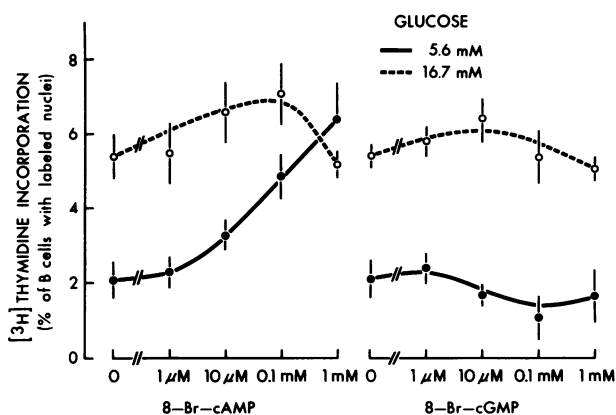
## DISCUSSION

There is now abundant evidence that cyclic nucleotides have a profound influence on cellular growth and differentiation in a variety of tissues (29). Possible inter-

actions of cyclic nucleotides in the growth and differentiation of pancreatic islet cells have not been examined in any detail. The studies of Pictet et al. (30) suggest that development of islet B cell insulin secretory competence in the fetal pancreas may be influenced by cyclic nucleotides. This group has also described a cAMP-dependent effect of a mesenchymal factor on DNA synthesis in embryonic pancreatic epithelial cell cultures (31). Maintenance of adult rat islets in culture medium with dibutyryl cAMP has been shown to increase the recovery of insulin in medium and islets after 8 d of culture (32). Also, the phosphodiesterase inhibitor, IBMX, has recently been reported to promote the formation of islet cell monolayer cultures from isolated rat islets (33).

The present study demonstrates that agents known to increase cellular levels of cAMP (IBMX, 8-Br-cAMP, and cholera toxin) promoted the survival of insulin-producing B cells in monolayer culture and significantly increased B cell replication, as measured by the [ $^3$ H]thymidine labeling index, as well as by the frequency of mitosis in these cells. The effects on B cell replication of adding 0.1 mM IBMX, 1 mM 8-Br-cAMP, or 0.1 nM cholera toxin to a low glucose concentration (5.6 mM) were similar to the effects observed by adding a high glucose concentration (16.7 mM) to the cultures. Since a high glucose concentration stimulates adenylate cyclase (5, 12) and increases cAMP levels in cultured islets (13), the present results suggest that cAMP may mediate, at least in part, the stimulatory effects of glucose on replication of insulin-producing B cells in tissue culture.

There is abundant evidence implicating cAMP as an important modulator of insulin secretion (34), and this study provides the first demonstration that cAMP can



**FIGURE 5** Dose-dependent effects of 8-Br-cAMP (1  $\mu$ M–1 mM) and 8-Br-cGMP (1  $\mu$ M–1 mM) on [ $^3$ H]thymidine incorporation in nuclei of islet B cells cultured for 12 d in medium containing 5.6 mM or 16.7 mM glucose. Each value is the mean  $\pm$  SEM for five culture dishes.

TABLE III  
Effects of Agents that Increase cAMP on Insulin Release and B Cell Replication

Additions to culture medium*			DNA†	cAMP‡	Insulin release <sup>§</sup>	B Cell replication¶
Glucose	IBMX	Cholera toxin				
mM			ng/8 dishes	pmol/8 dishes	ng/dish per 18 h	% Labeled B cells
5.6	0	0	423±50	1.88±0.15	49±4	2.9±0.3
5.6	0.1 mM	0	480±39	4.09±0.71	287±24	9.1±0.9
16.7	0	0	493±62	3.16±0.19	769±65	7.5±0.7
16.7	0.1 mM	0	543±58	7.65±0.14	1,067±46	8.7±0.5
5.6	0	0	—	1.36±0.17	141±22	2.9±0.2
5.6	0	0.1 nM	—	3.60±0.41	509±47	7.7±0.3
16.7	0	0	—	2.03±0.31	893±81	6.1±0.4
16.7	0	0.1 nM	—	5.57±0.87	1,009±87	6.5±0.2

\* Cultures were incubated for 2 d in media with additions as indicated.

† Cells were pooled from eight dishes for each DNA assay; mean values±SEM for three groups of eight dishes after 2 d of culture.

‡ Cells were pooled from eight dishes for each cAMP assay; mean values±SEM for three groups of eight dishes after 2 d of culture.

§ Insulin accumulation in medium during the last 18 h of a 2-d culture; mean values±SEM for 16–24 dishes.

¶ Cultures were incubated in 10 µCi/ml [<sup>3</sup>H]thymidine during the last 18 h of a 2-d culture; mean values±SEM for five to seven dishes.

also stimulate replication of islet B cells. However, the stimulatory effects of cAMP on islet B cell replication could be separated from its effects on insulin secretion. Thus, addition of 0.1 mM IBMX, or 0.1 nM cholera toxin, to 5.6 mM glucose produced slightly greater increases in cAMP levels and B cell replication than did 16.7 mM glucose, whereas insulin release was increased significantly more with 16.7 mM glucose (Table III). Furthermore, addition of 0.1 mM IBMX, or 0.1 nM cholera toxin, to 16.7 mM glucose stimulated further increases in cAMP levels and insulin release in the cultures, but no further increases in B cell replication. These results indicate that the increases in islet levels of cAMP obtained with IBMX or cholera toxin at a low (5.6 mM) glucose concentration are sufficient to stimulate B cell replication maximally, whereas insulin release is increased maximally only in the combined presence of agents such as IBMX or cholera toxin and a high (16.7 mM) glucose concentration. Thus, cAMP may serve as a sufficient mediator of B cell replication, in contrast to its secondary role as an amplifier of insulin release primarily mediated by actions of glucose that are independent of cAMP (34).

A dissociation between the effects of cAMP on B cell replication and insulin release was also reported by King et al. (11), who found that high concentrations of theophylline (≥1 mM) significantly decreased B cell replication in neonatal rat pancreatic monolayers cultured in the presence of a high (16.5 mM) glucose concentration. We also found that lower concentrations of IBMX (1 µM to 0.1 mM) decreased B

cell replication slightly in medium with 16.7 mM glucose, and 1 mM IBMX decreased B cell replication markedly (Fig. 3), however the latter effect was accompanied by toxic effects to the islet cells (Fig. 4). These observations highlight the importance of assessing effects of cyclic nucleotides on cell replication at lower, possibly more physiological concentrations.

It has been assumed in the present work that measurements of cAMP in the cultures reflect levels of this nucleotide in islet B cells. This is likely to be the case, since we have found that the proportion of B cells (aldehyde-thionine positive) in the monolayers averaged 88% (range, 82–92%), and this figure is in agreement with the value of 86% B cells in isolated islets of adult rats (35). Nevertheless, a possible contribution of other islet cell types to the measured levels of cAMP must be borne in mind.

In conclusion, we have demonstrated that agents that increase cAMP levels in pancreatic islet cell monolayer cultures also stimulate replication in the islet B cells. Because the effects of these agents (IBMX, 8-Br-cAMP, cholera toxin) on B cell replication were maximal at a low glucose concentration (5.6 mM), and since a high glucose concentration (16.7 mM) also increased the level of cAMP in the cultures, it is proposed that this cyclic nucleotide may mediate the stimulatory effect of glucose on B cell replication. Finally, since a limited ability of the islet B cell to replicate may contribute to the pathogenesis of diabetes mellitus (36), further study of mechanisms involved in cAMP-induced B cell replication appears warranted.

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## REFERENCES

1. Andersson, A., and C. Hellerstrom. 1972. Metabolic characteristics of isolated pancreatic islets in tissue culture. *Diabetes*. **21**: 546-554.
2. Andersson, A. 1974. Long-term effects of glucose on insulin release and glucose oxidation by mouse pancreatic islets maintained in tissue culture. *Biochem. J.* **140**: 337-382.
3. Buitrago, M., E. Gylfe, B. Hellman, L. A. Idahl, and M. Johansson. 1975. Function of microdissected pancreatic islets cultured in a chemically defined medium. I. Insulin content and release. *Diabetologia*. **11**: 535-540.
4. Frankel, B. J., E. Gylfe, B. Hellman, and L. A. Idahl. 1976. Maintenance of insulin release from pancreatic islets stored in the cold for up to 5 weeks. *J. Clin. Invest.* **57**: 47-52.
5. Lacy, P. E., E. H. Finke, S. Conant, and S. Naber. 1976. Long-term perfusion of isolated rat islets in vitro. *Diabetes*. **25**: 485-493.
6. Chick, W. L., V. Lauris, J. H. Flewelling, K. A. Andrews, and J. M. Woodruff. 1973. Effects of glucose on beta cells in pancreatic monolayer cultures. *Endocrinology*. **92**: 212-218.
7. Andersson, A., J. Westman, and C. Hellerstrom. 1974. Effects of glucose on the ultrastructure and insulin biosynthesis of isolated mouse pancreatic islets maintained in tissue culture. *Diabetologia*. **10**: 743-753.
8. Chick, W. L. 1973. Beta cell replication in rat pancreatic monolayer cultures: effects of glucose, tolbutamide, glucocorticoid, growth hormone and glucagon. *Diabetes*. **22**: 687-693.
9. Andersson, A. 1975. Synthesis of DNA in isolated pancreatic islets maintained in tissue culture. *Endocrinology*. **96**: 1051-1054.
10. King, D. L., and W. L. Chick. 1976. Pancreatic beta cell replication: effects of hexose sugars. *Endocrinology*. **99**: 1003-1009.
11. King, D. L., K. C. Kitchen, and W. L. Chick. 1978. Pancreatic B-cell replication: relation to insulin secretion. *Endocrinology*. **103**: 1321-1327.
12. Howell, S. L., I. C. Green, and W. Montague. 1973. A possible role of adenylate cyclase in the long-term dietary regulation of insulin secretion from rat islets of Langerhans. *Biochem. J.* **136**: 343-349.
13. Rabinovitch, A., G. S. Cuendet, G. W. G. Sharp, A. E. Renold, and D. H. Mintz. 1978. Relation of insulin release to cyclic AMP content in rat pancreatic islets maintained in tissue culture. *Diabetes*. **27**: 766-773.
14. Pawelek, J., R. Halaban, and G. Christie. 1975. Melanoma cells which require cyclic AMP for growth. *Nature (Lond.)*. **258**: 539-540.
15. Short, J., K. Tsukada, W. A. Rudert, and I. Lieberman. 1975. Cyclic adenosine 3':5' monophosphate and the induction of deoxyribonucleic acid synthesis in liver. *J. Biol. Chem.* **250**: 3602-3606.
16. Green, H. 1978. Cyclic AMP in relation to proliferation of the epidermal cell: a new view. *Cell*. **15**: 801-811.
17. Lambert, A. E., B. Blondel, Y. Kanazawa, L. Orci, and A. E. Renold. 1972. Monolayer cell culture of neonatal rat pancreas. Light microscopy and evidence for immunoreactive insulin synthesis and release. *Endocrinology*. **90**: 239-248.
18. Chick, W. L., A. A. Like, and V. Lauris. 1975. Pancreatic beta cell culture: preparation of purified monolayers. *Endocrinology*. **96**: 637-643.
19. Scott, D. A., and A. M. Fisher. 1938. The insulin and zinc content of normal and diabetic pancreas. *J. Clin. Invest.* **17**: 725-728.
20. Herbert, V., K. S. Lau, C. W. Gottlieb, and S. J. Bleicher. 1965. Coated charcoal immunoassay of insulin. *J. Clin. Endocrinol. Metab.* **25**: 1375-1384.
21. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-131 labeled human growth hormone of high specific activity. *Nature (Lond.)*. **194**: 495-496.
22. Paget, G. E. 1959. Aldehyde-thionin: a stain having similar properties to aldehyde-fuchsin. *Stain Technol.* **34**: 223-226.
23. Caro, L. G., R. P. Van Tubergen, and J. A. Kolb. 1962. High-resolution autoradiography. I. Methods. *J. Cell Biol.* **15**: 173-188.
24. Worton, R. G., and C. Duff. 1979. Karyotyping. *Cell Culture. Methods Enzymol.* **58**: 322-344.
25. Kissane, J. M., and E. Robbins. 1958. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. *J. Biol. Chem.* **233**: 184-188.
26. Green, I. C., and K. W. Taylor. 1972. The effects of pregnancy on rat islets. *Endocrinology*. **54**: 317-325.
27. Frandsen, E. K., and G. Krishna. 1976. A simple ultrasensitive method for the assay of cyclic AMP and cyclic GMP in tissues. *Life Sci.* **18**: 529-541.
28. Schultz, G., J. G. Hardman, K. Schultz, J. W. Davis, and E. W. Sutherland. 1973. A new enzymatic assay for guanosine 3':5'-cyclic monophosphate and its application to the ductus deferens of the rat. *Proc. Natl. Acad. Sci. U. S. A.* **70**: 1721-1725.
29. Friedman, D. L. 1976. Role of cyclic nucleotides in cell growth and differentiation. *Physiol. Rev.* **56**: 652-703.
30. Pictet, R. L., W. R. Clark, R. H. Williams, and W. J. Rutter. 1972. An ultrastructural analysis of the developing embryonic pancreas. *Dev. Biol.* **29**: 436-443.
31. Filosa, S., R. Pictet, and W. J. Rutter. 1975. Positive control of cyclic AMP on mesenchymal factor controlled DNA synthesis in embryonic pancreas. *Nature (Lond.)*. **257**: 702-704.
32. Ziegler, B., M. Ziegler, and R. Mehling. 1975. Effect of dibutyryl cyclic AMP on glucagon and insulin storage and secretion in organ culture of rat islets. *Experientia (Basel)*. **31**: 610-612.
33. Ohgawara, H., R. Carroll, C. Hofmann, C. Takahashi, M. Kikuchi, A. Labrecque, Y. Hirata, and D. F. Steiner. 1978. Promotion of monolayer formation in cultured whole pancreatic islets by 3-isobutyl-1-methylxanthine. *Proc. Natl. Acad. Sci. U. S. A.* **75**: 1897-1900.
34. Sharp, G. W. G. 1979. The adenylate cyclase-cyclic AMP system in islets of Langerhans and its role in the control of insulin release. *Diabetologia*. **16**: 287-296.
35. Reaven, E. P., G. Gold, and G. M. Reaven. 1979. Effect of age on glucose-stimulated insulin release by the  $\beta$ -cell of the rat. *J. Clin. Invest.* **64**: 591-599.
36. Gepts, W., and J. De Mey. 1978. Islet cell survival determined by morphology. *Diabetes*. **27**(Suppl. 1): 251-261.