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Stimulation of Cyclic AMP Secretion in Vero Cells by Enterotoxins of *Escherichia coli* and *Vibrio cholerae*

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The morphological response of Vero cells to *Escherichia coli* heat-labile enterotoxin was similar to that of cholera toxin and was accompanied by increases in the intracellular level of cyclic AMP. The effects of both enterotoxins were enhanced by the presence of phosphodiesterase inhibitor and inhibited by heat or specific antisera. Accumulation of cyclic AMP preceded the morphological response.

In a previous report (15) we showed that the continuous cell line Vero (African green monkey kidney) responded to culture filtrates of enterotoxigenic *Escherichia coli* with characteristic morphological changes; the responsible agent was heat labile. Vero cells compared favorably in sensitivity with Chinese hamster ovary (CHO) and Y-1 (mouse adrenal tumor) cells and were the simplest and most economical to use.

It has been reported that heat-labile *E. coli* enterotoxin or cholera toxin (CT) stimulates adenylate cyclase activity in CHO (10) and Y-1 (6) cells. Morphological responses in these cells were accompanied by increased production of intracellular cyclic AMP (cAMP) and Δ^4,3-ketosteroids, respectively. Numerous other cell or tissue cultures such as thymocytes (1, 19), thyroid slices (14), intestinal epithelial cells (11), and myocardial tissue (7) responded to these enterotoxins with an increase of cAMP but without apparent morphological response.

In this study we examined whether the morphological changes in Vero cells were accompanied by changes in the intracellular production of cAMP. We also compared the effects of *E. coli* enterotoxin with those of purified CT.

MATERIALS AND METHODS

Materials. Purified CT (lot CZ2785) was obtained from Schwarz/Mann (Orangeburg, N.Y.), purified cholera antitoxin (8940 A.T., Swiss Serum and Vaccine Institute) was from C. E. Miller (National Institute of Allergy and Infectious Diseases, Bethesda, Md.), 3-isobutyl-1-methylxanthine (MIX) was from Aldrich Chemical Co. (Milwaukee, Wis.), cAMP radioimmunoassay kit was from New England Nuclear (Boston, Mass.), and polyethylene glycol (6000) was from Bio Nuclear Products Ltd. (Ottawa, Ont.).

Enterotoxin production. Three enterotoxin-producing and two control strains of *E. coli* described previously (15) and strain TD214C_1 (producer of heat-stable enterotoxin only) obtained from J. G. Wells (Atlanta, Ga.) were used in this investigation. They were inoculated into 20 ml of tryptic soy broth (Difco) in 250-ml Erlenmeyer flasks and shaken on a rotary shaker (200 rpm) at 35°C. After 18 h, the cultures were centrifuged at 17,000 × *g* for 30 min, and the supernatants were filtered through 0.45-μm membrane filters (Millipore Corp., Bedford, Mass.). The filtrates (dry weight, 24.5 ± 0.1 mg/ml) were prepared on the day of each experiment. Filtrate of strain H10407 (ECF) was diluted with tryptic soy broth, and its concentration was expressed as dilution of the original filtrate.

For the preparation of partially purified heat-labile toxin (ECT), strain H10407 was grown in Evans medium (8) by a modification of the "sac culture" method (16). The culture supernatant was concentrated with a hollow-fiber concentrator, model CH3, with an H1P10 Diaflo cartridge (Amicon Corp., Lexington, Mass.). The concentrate was washed with water, lyophilized, and stored at −20°C. This preparation contained 16% protein as determined by the method of Folin-Ciocalteu (3). For assay, ECT was dissolved in saline.

Measurement of intracellular concentration of cAMP. Vero cells were cultured as described previously (15). Monolayers were prepared from stock culture by seeding 10^6 cells per 5 ml of medium 199 containing 10% fetal calf serum, in 60- by 15-mm plastic dishes (Falcon), 48 h prior to assay. At the time of assay the medium was exchanged with 2 ml of fresh medium 199 containing 1% fetal calf serum and 0.05 mM MIX, unless stated otherwise. The plates were incubated for 30 min before the addition of 0.2 ml of toxin preparation. Monolayers were incubated at 36°C in a 5% CO_2 atmosphere for 24 h, unless stated otherwise, and were examined for morphological change at the indicated incubation times. The cell response was recorded as 1, 2, 3, or 4, corresponding roughly to ≤25, 50, 75, or ≥90% cells affected, respectively.

For cAMP determination, the growth medium was removed, cells were washed with cold saline, and cold 5% trichloroacetic acid was added (9). After centrifugation, residual trichloroacetic acid was extracted four

times with ethyl ether saturated with water. Samples were then lyophilized and suspended in 0.05 M sodium acetate buffer, pH 6.2. cAMP was measured by radioimmunoassay according to the procedure provided by the manufacturer of the cAMP radioimmunoassay kit based on the method of Steiner et al. (17). Due to inadequate separation of bound and free antigen, the procedure was slightly modified by the addition of cold 2% polyethylene glycol in saline to each tube after overnight incubation (4). The tubes were then kept at 4°C for 10 to 15 min and centrifuged at 3,000 × *g* for 15 min, and the supernatant was aspirated.

The trichloroacetic acid precipitate was dissolved in 0.2 N NaOH, and the protein content was determined by the method of Folin-Ciocalteau (3), with bovine serum albumin (Sigma) as a standard. Concentrations of cAMP are expressed as picomoles of cAMP per milligram of cellular protein. Assays were performed in triplicate with at least two separate experiments.

Toxin neutralization and heat treatment. The preparation of antiserum to *E. coli* H10407 filtrate was described elsewhere (12). Antiserum to partially purified toxin (ECT) from the same strain was prepared essentially as described for the filtrate (12). The relative potencies of these antisera and of cholera antitoxin, determined by morphological response after mixing twofold dilutions 1:1 for 1 h at 35°C with ECF (undiluted), ECT (2.5 mg/ml), and CT (1 μg/ml), are given in Table 1. For cAMP determination, undiluted antisera against ECF and ECT and a 1:10 dilution of cholera antitoxin were used to neutralize ECF, ECT, and CT. For heat treatment, ECF, ECT, and CT of the same concentrations as above were heated at 100°C for 15 min.

RESULTS

Culture filtrates from several strains of *E. coli* were examined for stimulation of cAMP after 24 h in Vero cells. Strain H10407 showed the greatest increase of cAMP over the control level (Table 2). There was no cAMP increase or morphological response with filtrate of strain TD214C_1 (producer of heat-stable toxin only) or the nontoxigenic strains 711 and K-12. Culture filtrates of strains H10407, B7A, and P155 had no effect when pretreated with antisera against ECF or ECT of strain H10407.

Vero cells responded to CT in the same manner as to *E. coli* heat-labile enterotoxin; the affected cells were enlarged, thick walled, and refractile (15). ECF, ECT, and CT elicited dose-dependent increases in cAMP and morphological response; these were potentiated with MIX (Tables 3, 4, and 5). The smallest doses to show stimulation and response in the presence and absence of MIX after 24 h were: with ECF, dilutions of 1/64 and 1/16; with ECT, 0.6 and 2.5 mg/ml; and with CT, 10 and 100 ng/ml. Morphological response was noted with doses which stimulated cAMP to levels >2× the control levels; maximum morphological response was found at levels >3.5× the control levels. (The control levels of cAMP varied between experiments; therefore, the absolute levels of cAMP could not be well correlated with the extent of morphological response.) Preparations that were heated or treated with antisera to ECF, ECT, or CT showed no increase in cAMP or morphological response.

The time course of cAMP accumulation and morphological response with ECF, ECT, and CT is shown in Table 6. Increase in cAMP was evident at 120, 60, and 30 min, respectively, and preceded morphological response. The maximum levels of cAMP within a 24-h period were found at 24, 4, and 2 h, respectively.

TABLE 1. *Neutralization of morphological response in Vero cells to ECF (undiluted), ECT (2.5 mg/ml), and CT (1 μg/ml)*

Antiserum to:	Reciprocal of the highest dilution of antisera to neutralize cell response[a]		
	ECF	ECT	CT
ECF	32	2	0
ECT	64	4	2
CT	32	16	128

[a] Readings were recorded 24 h postincubation.

DISCUSSION

The present study demonstrated that the morphological response of Vero cells to *E. coli* heat-labile enterotoxin and CT was accompanied by increased levels of cAMP. Both parameters showed a dose-dependent increase; morphological response was obtained only with concentrations of toxins adequate to induce increased levels of cAMP. In the absence of MIX, cAMP levels with the three toxin preparations were lower and the morphological response was relatedly reduced. Guerrant et al. (10) obtained similar effects with MIX on CHO cells treated with these toxins. The increase in effect with MIX is apparently due to its inhibition of phosphodiesterase (2); Sutherland and Rall (18) observed that this enzyme, present in virtually all tissues, rapidly inactivated cAMP.

The time course study demonstrated that accumulation of cAMP preceded the morphological response, suggesting that activation of adenylate cyclase and accumulation of cAMP are prerequisite to the morphological response. A similar temporal relationship between morphological response and activation of the protein kinase was found in CHO cells exposed to CT (13).

In a comparative study of morphological response with other cell lines, we found that Vero cells were more sensitive than Y-1 cells but less sensitive than CHO cells when treated with cul-

TABLE 2. *Accumulation of cAMP and morphological response in Vero cells treated for 24 h with filtrates of toxigenic and nontoxigenic E. coli strains*

Strain	Serotype	cAMP[a] (pmol/mg of protein)	Increase[b]	Morphological response[c]
H10407	O78:K2	52.4 ± 4.4	5.95	4
B7A	O148:H28	41.5 ± 5.6	4.71	4
P155	O149:K91:88*ac*	33.0 ± 3.7	3.75	4
TD214C_1	Nontypable	8.5 ± 1.1	0.96	0
711	O18*ab*:K?:H14	8.7 ± 1.3	0.98	0
K-12	Not known	9.3 ± 0.96	1.05	0
Tryptic soy broth medium (control)		8.8 ± 0.79	1.00	0

[a] Each value represents the mean ± standard error.
[b] Ratio of mean cAMP level to control level.
[c] 0, No response; 4, ≥90% cells affected.

TABLE 3. *Correlation between cAMP level and morphological response in Vero cells treated with different dilutions of ECF with and without MIX for 24 h*

ECF (reciprocal dilution)	With MIX			Without MIX		
	cAMP[a] (pmol/mg of protein)	Increase[b]	Morphological response[c]	cAMP (pmol/mg of protein)	Increase	Morphological response
0	6.2 ± 0.9	1.0	0	4.8 ± 0.7	1.0	0
512	6.1 ± 1.8	1.0	0	4.7 ± 1.5	1.0	0
64	12.8 ± 0.6	2.1	1	5.2 ± 1.2	1.1	0
16	16.3 ± 4.1	2.6	2	10.0 ± 3.5	2.1	1
4	23.8 ± 3.5	3.8	4	15.1 ± 2.8	3.1	3
1	44.9 ± 5.4	7.2	4	24.3 ± 5.1	7.2	4

[a] Mean ± standard error.
[b] Ratio of mean cAMP level to control level.
[c] 0, No response; 1, ≤25%; 2, ca. 50%; 3, ca. 75%; 4, ≥90% cells affected.

TABLE 4. *Correlation between cAMP level and morphological response in Vero cells treated with different concentrations of ECT with and without MIX for 24 h*

ECT (mg/ml)	With MIX			Without MIX		
	cAMP[a] (pmol/mg of protein)	Increase[b]	Morphological response[c]	cAMP (pmol/mg of protein)	Increase	Morphological response
0	12.8 ± 1.6	1.0	0	11.5 ± 0.9	1.0	0
0.6	33.6 ± 6.1	2.6	1	18.4 ± 1.3	1.6	0
2.5	62.1 ± 3.2	4.8	4	32.1 ± 2.8	2.8	1
5.0	70.3 ± 6.1	5.5	4	40.5 ± 4.2	3.5	3
10.0	102.1 ± 5.8	7.9	4	49.8 ± 3.8	4.3	4

[a] Mean ± standard error.
[b] Ratio of mean cAMP level to control level.
[c] 0, No response; 1, ≤25%; 2, ca. 50%; 3, ca. 75%; 4, ≥90% cells affected.

TABLE 5. *Correlation between cAMP level and morphological response in Vero cells treated with different concentrations of CT with and without MIX for 24 h*

CT (ng/ml)	With MIX			Without MIX		
	cAMP[a] (pmol/mg of protein)	Increase[b]	Morphological response[c]	cAMP (pmol/mg of protein)	Increase	Morphological response
0	13.1 ± 1.7	1.0	0	11.6 ± 0.92	1.0	0
1.0	24.1 ± 3.3	1.8	0	22.3 ± 1.1	1.9	0
10.0	30.3 ± 5.2	2.3	2	21.8 ± 2.5	1.8	0
10^2	60.8 ± 3.2	4.6	4	33.8 ± 7.2	2.9	2
10^3	132.3 ± 6.8	10.1	4	60.4 ± 1.9	5.2	4
10^4	116.0 ± 10.6	8.8	4	40.7 ± 3.3	3.5	4

[a] Mean ± standard error.
[b] Ratio of mean cAMP level to control level.
[c] 0, No response; 2, ca. 50%; 4, ≥90% cells affected.

TABLE 6. *Time course of cAMP accumulation and morphological response in Vero cells exposed to ECF (undiluted), ECT (2.5 mg/ml), and CT (1 μg/ml)*

Time (h)	ECF		ECT		CT	
	cAMP[a] (pmol/mg of protein)	Morphological response[b]	cAMP (pmol/mg of protein)	Morphological response	cAMP (pmol/mg of protein)	Morphological response
0	8.8 ± 1.1	0	6.2 ± 0.7	0	10.5 ± 0.8	0
0.5	8.9 ± 0.9	0	6.4 ± 0.9	0	70.0 ± 1.2	0
1.0	9.3 ± 1.3	0	22.8 ± 1.7	0	165.2 ± 7.0	1
2.0	19.0 ± 2.9	0	82.3 ± 3.2	2	180.0 ± 18.2	4
4.0	31.3 ± 3.3	4	113.2 ± 2.8	4	117.8 ± 10.4	4
6.0	39.1 ± 4.6	4	87.6 ± 3.8	4	102.2 ± 8.9	4
12.0	47.5 ± 4.4	4	83.2 ± 5.1	4	101.4 ± 9.7	4
24.0	51.2 ± 1.8	4	65.3 ± 6.6	4	92.3 ± 7.9	4

[a] Mean ± standard error.

[b] 0, No response; 1, ≤25%; 2, ca. 50%; 4, ≥90% cells affected.

ture filtrates of several toxigenic strains of *E. coli* (15). Although it was not the intention of the present study to compare sensitivities, it is interesting to note that Vero cells appear to be the least sensitive to CT as measured by cAMP response. Maximum accumulation of cAMP was obtained in Vero cells with 1,000 ng/ml and in CHO cells with 100 ng/ml (10); in Y-1 cells maximum secretion of Δ^4,3-ketosteroids occurred with 1 to 5 ng of CT per ml (5).

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