Calcitonin-Responsive Clonal Cell Line from Porcine Kidney (PK(15)) in Serum-Free Medium

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Abstract

PK(15), a homogeneous epithelial cell line from porcine kidney which was originally established through single cell cloning from PK-2a, was found to respond to [125 I]calcitonin with an increase in adenosine 3':5'-cyclic monophosphate (cAMP) content, but do not respond to parathyroid hormone or arginine vasopressin.

These cells were able to grow in a synthetic medium (a 1:1 mixture of Dulbecco's modified Eagle's MEM and Ham's F12 medium) without any supplementary factor. The medium supplemented with selenous acid, transferrin, and insulin permitted a growth rate equivalent to those in serum containing medium. When grown in the serum-free defined medium, these cells showed an increase in cAMP content in response to [125 I]calcitonin to approximately the same degree as in the serum containing medium (10% fetal calf serum). Our present study first indicates that PK(15) cells are capable of growing in the serum-free defined medium retaining the calcitonin responsiveness of the original cells.

Several peptide hormones are known to increase adenosine 3':5'-cyclic monophosphate (cAMP) content in the renal tubules and also in several cell lines derived from kidneys (Ishizuka *et al.*, 1978; Goldring *et al.*, 1978; Rindler *et al.*, 1979; Teitelbaum and Strewler, 1984). These cell lines have been widely used for the study of hormone actions on renal tubular cells. However, it is possible that each cell line is a mixture of several cells with different biological characteristics (Handler *et al.*, 1980). They might also change the population of their component cells during the culture in different conditions (Takaoka and Katsuta,

1971). Thus, for the study of hormone actions on cells, a single kind of cell from a single cell cloning is considered to be most suitable.

In the current study, cAMP responsiveness to hormones of PK(15) cells, which were established through single cell cloning from PK-2a, was investigated and compared with that of primary culture cells from C57BL/6J mouse kidney. The growth of PK(15) cells was also studied in the serum-free defined medium with several growth factors, and this is considered to be useful in studying the hormone action without any interference from numerous undefined substances contained in the serum (Barnes and Sato, 1980). Also, the requirement of growth factors for

the proliferation of PK(15) cells was compared with that of MDCK cells.

Materials and Methods

Cell culture

PK(15) cells (ATCC, CCL-33) and MDCK cells (ATCC, CCL-34) were purchased from Flow Laboratories Inc. (Virginia, U.S.A.) through Dai-nippon Seiyaku Co., Ltd. (Suita, Japan). The cells were seeded in 25 cm² culture flasks (Corning, U.S.A.) (1×10^5 cells/flask) using a 1:1 mixture of Dulbecco's modified Eagle's MEM and Ham's F12 medium (SFFD) supplemented with 10% fetal calf serum (FCS) (GIBCO). The culture medium was changed every 3 days and subculture was performed every week.

To obtain the primary culture cells of mouse kidney, we used the modified method previously reported by Fukase *et al.* (1982). Briefly, three- to five-week old C57BL/6J normal male mice were anesthetized with ether. Kidneys were removed and papillae were dissected from the decapsulated kidneys. The remaining tissue was minced into pieces one mm in size. The tissue from four kidneys was suspended in a 1 mM calcium supplemented Hanks' solution with 3 mg/ml collagenase (Type II Worthington), and 4 mg/ml hyaluronidase (Type 1-S, Sigma), agitated in a metabolic shaker for 11 min. The mixture was washed once with 40 ml of a 15% solution of FCS in Hanks' solution (calcium and magnesium free) and three times with Hanks' solution alone. Approximately 6×10^5 cells and tubule fragments in 30 ml of supplemented medium were added to 75 cm² culture flasks. The supplemented medium was a SFFD containing selenous acid (SA), transferrin (TF), insulin (INS), and prostaglandin E₁ (PGE₁) at the concentrations described in the following section. The medium was replaced every two days until the cells became confluent.

Serum-free Defined Medium

Serum-free defined medium was made by adding growth factors (SA 1.3 ng/ml, TF 5 μ g/ml, INS 5 μ g/ml, PGE₁ 50 ng/ml, hydrocortisone (HC) 1 nM, and epidermal growth factor (EGF) 20 ng/ml to SFFD. The dispersed cells with 0.05% trypsin-0.02% EDTA solution were washed once with SFFD containing 5% FCS and then washed four times with SFFD alone to remove FCS. The cells were inoculated on 20 cm² culture plates

(0.4×10^4 cells/cm²) after being resuspended in the various serum-free defined medium. The medium was changed every other day.

Hormone Stimulation

PK(15) cells were inoculated on 24 well culture plates (Corning) at 2×10^4 cells/cm² in 2 ml SFFD supplemented with 10% FCS or various growth factors. The medium was changed every other day. All experiments were performed at the time of subconfluency. After removing the culture medium, 0.5 ml of experimental medium (SFFD containing 1 mM isobutyl-methyl-xanthine) was added to each well. The culture plates were kept gently shaken for 60 min in a Dubnoff metabolic shaker at 37°C. Hormone solution (25 μ l) was then added to each well and incubation was performed for the appropriate time indicated in the "Results" at the 37°C in air. At the end of the incubation, the medium was transferred to ice-cooled tubes and 1 ml of 6% trichloroacetic acid (TCA) was added to each well. Medium and cells were immediately stored at -20°C before extraction and assay.

Primary culture cells obtained by the method described in "Cell Culture" were dispersed with trypsin-EDTA solution after reaching confluency (approximately 7th culture day). The cells (10^6 cells/well) were inoculated in 24 multiwell plates in a volume of 2 ml SFFD supplemented with SA, TF, INS, PGE₁, and 5% FCS. The medium was changed every two days. Experiments for hormone responses were performed on the 4th culture day by the method described above.

Alkaline Phosphatase Assay

The assay was performed as follows: 0.05 ml of sonicated cells was added to 3 ml of diethanolamine buffer solution (1.08 mM, pH 10.2), and the resultant mixture was allowed to equilibrate at 37°C for 5 min. The reaction was initiated by adding 0.1 ml of p-nitrophenyl phosphate stock solution (360 mM). After the initiation of the reaction, the absorbance of the samples at 405 nm was measured every minute for 5 minutes and p-nitrophenol concentrations were calculated from the increase in absorbance.

Cyclic AMP assay and Protein Measurements

The cells were subjected to freezing (at -20°C in the freezer) and thawing (at room temperature) three times, than scraped into 6% TCA with a rubber policeman and transferred to tubes. The

supernatant was collected after centrifugation at 3,000 rpm for 15 min and washed three times with diethyl ether to remove TCA. The supernatant was then kept in the vacuum drying oven (20 mmHg) at 20°C for three hours to remove diethyl ether and stored at -20°C before assay. The recovery of cAMP by this extraction method was calculated by using (8-³H) cAMP (Amersham). Each medium was assayed directly. Cyclic AMP content was measured by radioimmunoassay according to the method of Steiner *et al.* (1969) using an antiserum kindly provided by K. Martin (Martin *et al.*, 1978).

The protein content was measured by Lowry's method (Lowry, 1951).

Cell Counts

The cells dispersed with trypsin-EDTA were counted with a hemocytometer. Viable cells were determined by the method of trypan blue dye

exclusion.

Hormone Solution

Synthetic human parathyroid hormone(1-34) (hPTH(1-34)) and [¹²⁵I]calcitonin ([Asu^{1,7}]ECT), provided by Toyo Jozo Co., Ltd. (Shizuoka, Japan), were dissolved in 0.01 N HCl containing 0.2% BSA (bovine serum albumin Fraction, V, Sigma). Arginine vasopressin (AVP) and synthetic human calcitonin (hCT) were purchased from Protein Research Foundation (Minoh, Japan) and dissolved in 0.01 N HCl containing 0.2% BSA.

Results

Morphological Study

PK(15) cells had a polygonal epithelial-like appearance and were uniform in shape.

Fig. 1. Phase-contrast micrograph ($\times 100$) of PK(15) cells taken at 8 days' culture in the medium containing 10% FCS showing uniform appearance of epithelial cells which form multiple large "domes."

They formed many domes at the time of confluence (Fig. 1), if they were grown in serum containing medium or serum-free medium supplemented with SA, TF, INS, PGE₁.

Alkaline Phosphatase Activity

The alkaline phosphatase activity in PK(15) cells at the confluent state was 3.45 ± 0.20 nmol/min mg protein (Mean $\pm$ SE, $n=4$), whereas that in primary cultured mouse kidney cells, which are a mixture of cells originated from various nephron segments, was 18 times as great as that of PK(15) cells (62.6 ± 2.8 nmol/min mg protein, Mean $\pm$ SE, $n=5$).

Effect of Various Hormones on cAMP Levels

PK(15) cells were incubated with hPTH (1-34), [Asu^{1,7}]ECT, hCT or AVP, and intracellular cAMP levels were measured. Recovery of cAMP during extraction procedures was $84.1 \pm 2.6\%$ (Mean $\pm$ SE, $n=4$) by our extraction method. As shown in Fig. 2, [Asu^{1,7}]ECT and hCT increased the cAMP content dose-dependently in these cells, whereas hPTH(1-34) or AVP did not. The increase in cAMP content in response to [Asu^{1,7}]ECT was more prominent than that to hCT. On the other hand, primary culture cells from C57BL/6J mouse kidney increased the cAMP content in response to hPTH(1-34), [Asu^{1,7}]ECT, and AVP (Fig. 3).

Fig. 2. Dose-dependent relationship between cAMP content of PK(15) cells and concentrations of hPTH(1-34), [Asu^{1,7}]ECT, hCT, and AVP added in the medium. Incubations were performed on the subconfluent cells in 24 multiwell plates for 10 min at 37°C. Only calcitonin increased intra- and extracellular cAMP content. Vertical lines represent the Mean $\pm$ S.E. for four different wells. (●—●): intracellular cAMP. (○---○): cAMP content in the medium.

Fig. 3. Dose-dependent relationship between cAMP content of the primary cultured mouse kidney cells and concentrations of hPTH(1-34), [Asu^{1,7}]ECT, and AVP added in the medium. These cells increased cAMP content in response to all of these three hormones. Vertical lines represent the Mean $\pm$ S.E. for four different wells. (●—●): intracellular cAMP. (○---○): cAMP content in the medium.

The time course of these hormone effects on intra- and extracellular cAMP levels are demonstrated in Fig. 4. [Asu^{1,7}]ECT-stimulated cAMP increase in the cells became apparent at 5 min of test incubation, then decreased gradually, but did not return to the basal level during the 60 min when it was examined, while that in the medium rose slowly for 60 min.

Effect of Serum-Free Defined Medium on Cell Growth and cAMP Production in Response to [Asu^{1,7}]ECT

Growth of PK(15) and MDCK cells in

serum-free defined medium was studied (Fig. 5A, B). PK(15) cells were able to grow and increase in number in SFFD without any hormonal supplementation, and could proliferate in SFFD supplemented with SA, TF, and INS as rapidly as in the medium containing 10% FCS. In this defined medium, however, the plating efficiency was worse than in FCS-containing medium. Further addition of PGE₁, HC, EGF to the medium failed to enhance the cell proliferation (Table 1). In contrast with PK(15), MDCK cells could not proliferate in the serum-free medium without supplementation.

Fig. 4. Time course of effects of hPTH(1-34) ($1 \mu\text{g/ml}$), $[\text{Asu}^{1,7}]$ ECT ($1 \mu\text{g/ml}$), and AVP ($1 \mu\text{g/ml}$) on the cAMP production. Subconfluent cells were incubated with medium containing various hormones or solvent alone as control. After the various incubation times indicated on the abscissa, cAMP levels in cells ($\bullet$ — $\bullet$) and medium ($\circ$ — $\circ$), represented as $\text{pmol}/10^5$ cells, were measured. Vertical lines represent the $\text{Mean} \pm \text{S.E.}$ Determinations were made in four different wells in each experiment.

PK(15) cells, which grow to confluence in various serum-free media, were incubated with $[\text{Asu}^{1,7}]$ ECT for 5 min and cellular cAMP levels were measured (Table 2). Even when cultured in the serum-free defined media, the cAMP contents in PK(15) cells did increase in response to $[\text{Asu}^{1,7}]$ ECT dose-dependently as observed in the cells cultured in serum-containing medium. An increase in cAMP content was the most remarkable in the cells grown in the serum-free medium supplemented with SA, TF, and INS.

Discussion

Our present results indicate that PK(15) cells, obtained through single cell cloning, were able to grow in a synthetic medium without serum or hormone supplementation, and responded to $[\text{Asu}^{1,7}]$ ECT with an increase in cAMP content. In addition, the

proliferation of these cells in SFFD with SA, TF and INS was approximately equal to that in SFFD containing 10% FCS with retaining the responsiveness to $(\text{Asu}^{1,7})$ ECT.

The increase in cAMP contents in renal tubular cells on exposure to PTH, CT, or VP etc. has been well documented in various species (Chabardes *et al.*, 1975; Imbert *et al.*, 1975; Chabardes *et al.*, 1976; Brunette *et al.*, 1979; Chabardes *et al.*, 1980; and Kawashima *et al.*, 1981). A microdissection study revealed that calcitonin sensitive cells were located in the medullary ascending limb (MAL), cortical ascending limb (CAL) and distal convoluted tubules (DCT) of a rabbit kidney (Chabardes *et al.*, 1976), and in MAL, CAL, DCT, and collecting tubules of a rat kidney (Morel, 1981). Although there was no report about the distribution of calcitonin-sensitive adenylate cyclase along the porcine nephron, LLC-PK₁ cells (derived from porcine kidney) have been used as one

Fig. 5A, B. Effects of various media on PK(15) (Fig. 5A) and MDCK (Fig. 5B) cell growth. The cells were incubated at 8×10^4 cells per 20 cm² culture plate containing SFCD supplemented with 10% FCS (●—●); 1.3 ng/ml SA, 5 µg/ml TF, and 5 µg/ml INS (○—○); or no additional factors (×—×). PK(15) cells could proliferate in the serum-free medium without any supplementary factor, whereas MDCK could not. Determinations were made in triplicate. Vertical lines represents the Mean ± S.E.

Table 1. Effects of various media on the proliferation of PK(15) cells.

Medium	Doubling time (hours)
SFFD alone	47
SFFD with SA	32
SFFD with SA, TF	25
SFFD with SA, TF, INS	23
SFFD with SA, TF, INS, PGE ₁	23
SFFD with SA, TF, INS, PGE ₁ , HC	22
SFFD with SA, TF, INS, PGE ₁ , HC, EGF	23
SFFD with 10% FCS	23

PK(15) cells could proliferate in the serum-free media supplemented with various growth factors as rapidly as in the medium containing 10% FCS.

Table 2. Effects of various media on the responsiveness of cAMP to [Asu^{1,7}]ECT in PK(15) cells.

Media	Basal cAMP (pmol/mg protein)	Ratio of cAMP increase from the basal	
		[Asu ^{1,7}]ECT 10 ng/ml	[Asu ^{1,7}]ECT 200 ng/ml
SFFD alone	37.2± 4.0	4.0±0.9	*4.6±0.2
SFFD with SA	33.2± 8.2	5.1±0.5	5.9±0.5
SFFD with SA, TF	48.2±10.6	4.2±0.1	*5.1±0.2
SFFD with SA, TF, INS	35.8± 2.4	*5.5±0.7	*7.3±0.5
SFFD with SA, TF, INS, PGE ₁	65.0± 3.8	*3.5±0.3	*3.6±0.1
SFFD with SA, TF, INS, PGE ₁ , HC	52.2± 0.0	3.9±0.4	*5.0±0.1
SFFD with SA, TF, INS, PGE ₁ , HC, EGF	71.6± 6.8	*3.5±0.2	*4.6±0.5
SFFD with 10% FCS	53.4±10.2	4.5±0.1	6.0±0.3

* Significantly different from the corresponding values in a SFFD with 10% FCS group by Duncan's New Multiple Range Test ($P>0.01$).

Incubations were performed for 5 min on the subconfluent cells. Abbreviations used are explained in "Materials and Methods" section. Values are the Mean±S.E. for three separate wells.

of the cells presumably corresponding to those distal segments of renal tubules based on a positive responsiveness to calcitonin. However, it is still uncertain whether LLC-PK₁ is composed of homogeneous cells or is a mixture of several kinds of cell types (Goldring *et al.*, 1978). Nevertheless, the following indicate that this cell line is composed of heterogenous cells: Positive responsiveness to calcitonin and vasopressin, which LLC-PK₁ has, could be characteristics of the cells derived from the distal nephron segments (Goldring *et al.*, 1978). Specific glucose transport (Mullin *et al.*, 1979) and high levels of alkaline phosphatase and r-

glutamyl transpeptidase activities (Rabito *et al.*, 1984), which LLC-PK₁ also has, are evidences indicating that the cells originate in the proximal renal tubules.

On the other hand, PK(15) is a cell line established through a single cell cloning from PK-2a by M. Harris in 1957 (American Type Culture Collection, 1983). The cells are homogeneous in morphology and not PTH nor AVP, but calcitonin increased the cAMP content in the cells. In addition, alkaline phosphatase activity (3.45 ± 0.20 nmol/min mg protein), which is a marker enzyme of proximal renal tubules, was extremely low in comparison with that of

primary cultured mouse kidney cells (62.6 ± 2.8 nmol/min mg protein) or LLC-PK₁ cells (683 ± 17 nmol/min mg protein) (Carlos *et al.*, 1984). These data suggest that PK(15) might be composed of a single kind of cell which originated from the calcitonin-responsive cells of the distal nephron. Furthermore, PK(15) cells increased cAMP content more prominently in response to [Asu^{1,7}]ECT than in response to hCT, ECT has been reported to have a stronger biological potency than hCT (Orimo and Yamanouchi, 1977), therefore PK(15) cells could be useful as a model system for studying calcitonin action.

Since serum-free defined culture medium supplemented with growth factors can eliminate the unknown substances for experiments, this medium has come to be employed for studying the effects of a small amount of hormones on the cells as well as for detecting the substances secreted by cultured cells (Barnes *et al.*, 1980). In terms of renal cells, effects of several factors on cell growth has been studied precisely by using MDCK cells, and 5 substances (triiodothyronine, TF, INS, PGE₁ and HC) were shown to be necessary for MDCK cells to grow in the serum-free defined medium (Taub *et al.*, 1979). Our studies disclosed that PK(15) cells were able to grow in serum-free medium without any supplementary factor and retain the cAMP responsiveness to calcitonin. These characteristics of PK(15) cells could make it possible to study the renal tubular cells without interferences from the biologically active growth factors contained in the growth factor supplemented serum-free medium.

In conclusion, PK(15) is considered to be an ideal cell line for studying the action of calcitonin on the renal tubular cells because, unlike LLC-PK₁, this cell line is composed of a single kind of cells and grows and maintain its character in the serum-free defined medium without any supplementary factor.

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