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A CELL STRAIN CULTURED FROM PORCINE KIDNEY INCREASES CYCLIC AMP CONTENT UPON EXPOSURE TO CALCITONIN OR VASOPRESSIN

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SUMMARY: Cells originally dispersed from whole juvenile male Hampshire pig kidney and maintained in monolayer culture, increased cyclic AMP content in response to incubation with salmon calcitonin or antidiuretic hormone. Parathyroid hormone and epinephrine did not affect cyclic AMP content. The apparent K_m for arginine vasopressin in the porcine cells was 3.0 nM which is similar to the value obtained in single segments of rabbit kidney tubule. The apparent K_m for salmon calcitonin of 2.7 nM is higher than that reported for the rabbit nephron segments, but comparable to the K_m obtained in rat kidney homogenates. Exposure of the porcine cells to exogenous prostaglandin E_2 did not affect cyclic AMP responses to other hormones. In the cultured porcine kidney cells the pattern of hormone response is similar to that observed in nephron segments prepared from the medullary portion of the thick ascending limb of the loop of Henle, and these findings suggest that the porcine cells may be related to cells present in the medullary region of the kidney tubule.

INTRODUCTION: Several polypeptide hormones are capable of increasing adenylate cyclase activity and cyclic AMP content in kidney tissues (1-6). Studies of isolated renal tubule segments from rabbit kidney have revealed a pattern of adenylate cyclase response to hormones specific for different regions of the nephron (7-10). In order to study the interaction of peptide hormones with isolated kidney cells in culture, we have utilized a strain of trypsin-dispersed porcine kidney cells, LLC-PK_1, originally isolated and characterized by Hull *et al.* (11). These cells have been maintained in monolayer culture since 1958 and despite multiple passages, cell morphology and growth characteristics have remained constant (11). In this communication we report that the LLC-PK_1 cells increase cyclic AMP content after exposure to salmon calcitonin or antidiuretic hormone. Other hormones fail to increase cellular content of cyclic AMP. This pattern of hormone response is

ABBREVIATIONS: TCA, trichloroacetic acid; PTH, parathyroid hormone; SCT, salmon calcitonin; AVP, arginine vasopressin; LVP lysine vasopressin; PGE_2, prostaglandin E_2; pmoles, picomoles.

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comparable to that observed in the medullary portion of the thick ascending limb of the loop of Henle (7-10).

MATERIALS AND METHODS:

Cell Culture Procedures: The LLC-PK_1 cell strain was originally prepared from whole juvenile male Hampshire pig kidney (11). The cells which we used were provided by Dr. R.N. Hull and had been through more than 200 passages. Cells were carried in 10 cm diameter plastic petri dishes (Falcon) using Dulbecco's modification of Eagle's medium (Grand Island Biological Company [GIBCO]), supplemented with 10% fetal calf serum (Microbiological Associates), plus 100 units of penicillin and 100 µg streptomycin/ml (GIBCO). Cells were usually plated at $\sim$ 1 x 10^6 cells per dish and reached a density of $\sim$ 1-2 x 10^7 cells per dish within 5-7 days at 37°C, 95% air, 5% CO_2. At that stage the cells showed a tendency to pile up and exhibit the domes described by Hull *et al.* (11). The cells were dispersed with 0.25% trypsin-EDTA (GIBCO) at 37°C for 10 min. Samples of cells (5 x 10^5 cells) were transferred to wells (16 mm diameter) in plastic trays (Costar, 24 wells/tray) and incubated in growth medium (1 ml/well) for 24-48 hours before hormone experiments were begun.

Hormone Stimulation: After removal of the culture medium, the cells were incubated with the selected hormone in Dulbecco's phosphate buffered saline (GIBCO), supplemented with 0.25% bovine serum albumin (Pentex) and 0.1% glucose. Stock solutions of the hormones were diluted in this buffer just prior to incubation. After the appropriate incubation period at 37°C in air, trichloroacetic acid (TCA) at 2°C was added to the wells in a final concentration of 5-8%. The trays were kept at -30°C until cyclic AMP assays were performed. To prepare samples for assay, trays were brought to room temperature and the wells scraped with a plastic policeman. The contents were then transferred to plastic tubes, centrifuged for 20 min at 600 x g, and the cyclic AMP extracted from the supernatant solutions.

Cyclic AMP Assay: The TCA-supernatant solutions were placed on columns of Dowex 50 W-X4 resin (200-400 mesh, H^+ form) and cyclic AMP eluted as previously described (12). Cyclic AMP was then assayed using the radioimmunoassay kit (^{125}I) obtained from Schwarz/Mann.

DNA Assay: DNA content of the TCA cell pellets was determined using a modified fluorimetric micromethod (13).

Prostaglandin Assay: Prostaglandins in cell culture media were measured in Dr. D.R. Robinson's laboratory, Massachusetts General Hospital, utilizing an antiserum to PGE_2 (14) kindly provided by Dr. L. Levine of Brandeis University.

Hormone Solutions: Test solutions of the hormones were prepared by dissolving lyophilized samples of either bovine parathyroid hormone (PTH) or synthetic salmon calcitonin (SCT) (Armour, 5000 MRC units/mg, a gift of Dr. Henry T. Keutmann), in the incubation buffer. Bovine PTH (3000 units/mg), also a gift of Dr. Keutmann, was prepared from the TCA precipitate of bovine parathyroid glands and purified by gel filtration and ion-exchange chromatography on carboxymethyl-cellulose. The stock solutions of the antidiuretic hormones used were Pitressin, Parke-Davis, $\sim$ 50 I.U./mg, arginine vasopressin (AVP), Sigma, 367 I.U./mg, and lysine vasopressin (LVP), Sigma, 100 I.U./mg. Media containing prostaglandin E_2 (PGE_2) were prepared by diluting a stock solution of 1 mg/ml of PGE_2 (Upjohn) in ethanol in the buffer solution.

RESULTS: The cells in monolayer culture had an epithelial-like appearance with a single large round prominent nucleus. The morphologic features and growth characteristics in the culture medium were similar to those described by Hull *et al.* (11). Aggregation of some cells to form ring-like structures were com-

monly observed and were similar to the three-dimensional "domes" described by Hull *et al.* (11).

The porcine kidney cells were incubated in the presence of several hormones which affect cyclic AMP content in the mammalian kidney. The results obtained are shown in Table 1. All preparations of the cells tested increased the cyclic AMP content when exposed to SCT or Pitressin. At these relatively high hormone concentrations, there was an increase in cyclic AMP content in cells plus medium of ∿ 30 fold with SCT or Pitressin, but no significant change in cyclic AMP content in cells or medium exposed to PTH, PGE_2 or epinephrine. In other experiments the addition of the phosphodiesterase inhibitor, isobutylmethylxanthine, did not alter the pattern of hormone response, although the absolute levels of cyclic AMP in cells plus medium were increased in all instances.

The dose-dependent relationship between cyclic AMP content in cells plus medium and concentrations of SCT and Pitressin are illustrated in Fig. 1. The minimum concentration required to change the cyclic AMP content differed for the two hormones, indicating a greater sensitivity of the cells to SCT. The threshold value for increasing cyclic AMP content was 0.3 nM for SCT and 1.8 nM for Pitressin. The slopes of the linear portion of the log dose-responses for the two hormones differed; the slope for Pitressin was steeper than that for SCT. The concentrations of SCT and Pitressin which yielded half-maximal increases in cyclic AMP content differed for the two hormones and were 2.7 nM for SCT and 5.0 nM for Pitressin. When the threshold sensitivities to arginine or lysine vasopressin were compared they were identical, yielding values similar to those observed with Pitressin. The apparent K_m for arginine vasopressin was ∿ 3.0 nM which was comparable to the value obtained with Pitressin. The rises in intracellular cyclic AMP content which were observed when the cells were exposed to near maximal concentrations of Pitressin or SCT were accompanied by increases in cyclic AMP content in the medium (Table 2).

In the presence or absence of SCT or Pitressin, PGE_2 released into medium containing 10% fetal calf serum were just below the limit of sensitivity of

Table 1. Effect of Hormones on cyclic AMP Content in Cells Plus Medium in Pig Kidney Cells

Hormone	cyclic AMP picomoles/μg DNA
None	4.7 ± 0.8
Salmon calcitonin, 290 nM	163.7 ± 12.8
Pitressin, 180 nM	140.0 ± 7.8
Prostaglandin E_2, 3 μM	6.2 ± 1.2
Salmon calcitonin, 290 nM, plus Prostaglandin E_2, 3 μM	206.0 ± 13.6
Pitressin, 180 nM, plus Prostaglandin E_2, 3 μM	160.2 ± 7.8
Epinephrine, 10 μM	8.2 ± 1.2
Bovine parathyroid hormone, 238 nM	7.3 ± 1.2

Incubations were performed on cells in multiwell trays at 37°C for 10 min. Values shown are means ± standard error for three separate wells.

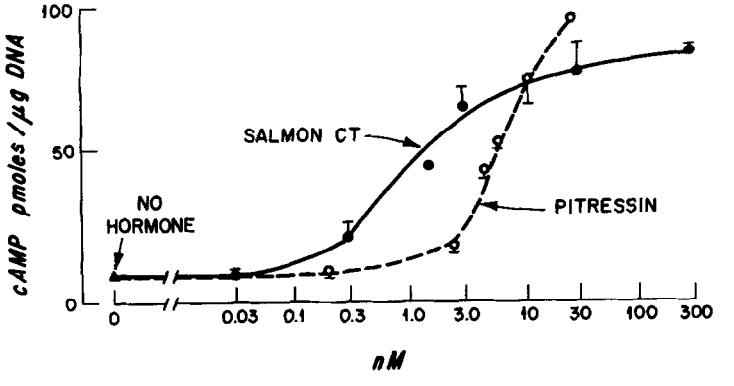

Fig. 1. The dose-dependent relationship between cyclic AMP (cAMP) content in cells plus medium and concentrations of salmon calcitonin (salmon CT) and Pitressin. Incubations were performed on cells in multiwell trays at 37°C for 10 min. Values shown are means ± standard error for three separate wells.

the assay method (< 1 ng/10^6 cells/day). In order to study the effect of exogenous PGE_2 on the cyclic AMP response to Pitressin and SCT, the cells were exposed to SCT (290 nM) and Pitressin (180 nM) in the presence or absence of PGE_2 (3 μM). The mean content of cyclic AMP in cells plus medium was higher in all cells exposed to PGE_2, but the differences were not statistically significant (Table 1).

Table 2. Effect of Hormones on cyclic AMP Content in Cells or Medium in Pig Kidney Cells

Hormone	cyclic AMP picomoles/μg DNA		
	Cells (A)	Medium (B)	B/A
None	4.0 ± 0.7	1.2 ± 0.2	0.3
Salmon calcitonin, 290 nM	48.7 ± 5.0	35.4 ± 8.1	0.7
Pitressin, 180 nM	55.0 ± 1.0	21.2 ± 0.8	0.4

Incubations were performed on cells in multiwell trays at 37°C for 10 min. Values shown are means ± standard error for three separate wells.

DISCUSSION: Parathyroid hormone, calcitonin, vasopressin and epinephrine increase adenylate cyclase activity in homogenates or in membrane fractions prepared from renal tissue (1-6). In order to localize the specific region of the nephron which is responsive to each of these hormones, micromethods have been developed for dissection of the rabbit kidney tubule and characterization of the pattern of adenylate cyclase response in single nephron segments (7-10). As is shown in Table 3, the sensitivity to hormone stimulation of adenylate cyclase differs in each segment of the renal tubule (7-10). We have shown that cells derived from porcine kidney and grown in monolayer culture increase cyclic AMP content when exposed to Pitressin or SCT, but fail to increase cyclic AMP content when incubated with epinephrine or PTH. The pattern of hormone response in the porcine cells is similar to that observed in nephron segments prepared from the medullary portion of the thick ascending limb of the loop of Henle and these findings suggest that the LLC-PK_1 cells may be related to cells present in the medullary region of the kidney tubule.

In order to investigate further the hormone response in the porcine kidney cells, the effect of increasing concentrations of Pitressin, LVP, AVP, and SCT were studied. The concentrations of AVP and SCT which produced half maximal increases in cyclic AMP content in cells plus medium were 3.0 nM and 2.7 nM respectively. The apparent K_m for AVP in the porcine cells was similar to the value

Table 3. Hormone Stimulation of Adenylate Cyclase in Rabbit Nephron Segments

	PCT	TDL	MAL	CAL	DCT	CCT	MCT
Vasopressin	-	-	+	+	-	+	+
Salmon calcitonin	-	-	+	+	+	-	-
Epinephrine	-	-	-	-	+	+	+
Parathyroid hormone	+	-	-	+	+	-	-

Distribution of hormone sensitive adenylate cyclase activity in specific segments of the rabbit nephron (7-10). Sensitivity of the adenylate cyclase to hormone stimulation is indicated by (+); lack of sensitivity is indicated by (-). PCT: proximal convoluted tubule; CAL: thick ascending limb (cortical); DCT: distal convoluted tubule; CCT: collecting tubule (cortical); MCT: collecting tubule (medulla).

(5 nM) obtained in studies of single segments of the rabbit kidney tubule (9). Although the apparent K_m of the porcine cells to SCT was higher than that reported for the rabbit nephron segments (0.07-0.11 nM) it is comparable to the values obtained in rat kidney homogenates (15). It is possible that these different K_m's for SCT are related to variations in hormone sensitivity in the individual species or alternatively may reflect differences in the *in vitro* systems employed.

The apparent uniformity of cell morphology observed with phase contrast optics and the consistency in the growth characteristics of these cells in monolayer culture through multiple passages suggest that the LLC-PK_1 cells represent a homogeneous cell population. In addition, in a single experiment (not shown) incubation of the cells with both AVP and SCT did not increase cyclic AMP content above values obtained in cells exposed to maximal effective concentrations of each hormone alone. These observations, however, do not exclude the possibility that the porcine cells consist of a heterogeneous cell population. After many passages in some experiments we have observed a decrease in the magnitude of the cyclic AMP response to maximal doses of Pitressin. SCT responses have not changed. The decrease in the magnitude of the cyclic AMP response to Pitressin may reflect changes in the composition of a heterogeneous cell population or could reflect altered hormone responsiveness in the same cell. Our initial attempts to clone these cells, however, have been unsuccessful.

Because Pitressin increases prostaglandin production by renal medullary cells (16) we investigated the effects of this hormone on prostaglandin release by the porcine kidney cells. We also studied the effects of PGE_2 on cyclic AMP response to Pitressin since changes in prostaglandin concentration may modulate the effects of this hormone on cyclic AMP content (5). The pig kidney cells did not produce detectable prostaglandins even after prolonged incubation with Pitressin. Furthermore, incubation with PGE_2 did not affect the cyclic AMP response to Pitressin or SCT, at least at the concentration of PGE_2 used. Whether these cells are capable of producing prostaglandins under other conditions remains to be established.

Since the porcine kidney cell cultures retain the ability to increase cyclic AMP content in response to specific hormones and since these cells can be easily grown in monolayer culture, they provide a potential *in vitro* system for investigating the mechanism of hormone-cell interactions in renal tissues.

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