Difference in Arginine Vasopressin Responsive cAMP Production Between Apical and Basolateral Membrane of Cultured Renal Epithelial Cells (MDCK)

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Abstract

It has been reported that vasopressin (AVP)-sensitive renal epithelial cell line (MDCK) forms morphologically polarized monolayers when cultured on plates. We studied whether the AVP-responsive cAMP production system would be located solely on the basolateral surface of these cells as has already been shown on the renal tubules. We used two methods to overcome the inaccessibility to the basolateral surface of the cultured cell layer and to study the apical and basolateral surfaces separately. One was culture on collagen sheet and the other was on Millipore filters. Our experiments showed that MDCK cell increased adenosine 3':5'-cyclic monophosphate (cAMP) content prominently only when vasopressin was accessible to the basolateral surface. Accordingly, MDCK cells were shown to have the AVP-responsive cAMP production system predominantly on the basolateral surface of the cell membrane.

Renal tubular epithelial cells are organized into tubules in vivo. They respond to various peptide hormones by increasing adenosine 3':5'-cyclic monophosphate (cAMP) and transport fluid and ions from the lumen to the blood. As peptide hormones are present in the blood, it is likely that hormone receptor-adenylate cyclase complexes are present on the basolateral surface of the cells. In fact, vasopressin (AVP) receptors were reported to be present on the basolateral surface of the renal tubular cells (Schwartz et al., 1974). MDCK cell line, which was originally isolated from whole adult dog kidney, forms a continuous sheet of transporting epithelial cells in a unilateral direction and increases cAMP content when exposed to AVP (Ishizuka et al., 1978; Rindler et al., 1979). The apical surface, studded with microvilli, is considered to be the luminal side (Misfeldt et al., 1976; Khayim et al., 1981). When cultured on a freely permeable membrane filter, MDCK cells form an epithelial membrane which transports water in an apical-to-basolateral direction and has transepithelial electrical potential with the apical surface negative (Misfeldt et al., 1976; Cereijido et al., 1978). These characteristics are similar to those of the renal tubules and have been used as a model of transporting renal tubules. However, no study of an asymmetrical distribution
of AVP-responsive cAMP production between the apical and basolateral surfaces of the monolayer reconstructed by MDCK cells has yet been made.

We investigated if the AVP-responsive cAMP production systems could be distributed asymmetrically. Two different experimental procedures were used to overcome the inaccessibility of the basolateral surface of the cell layer and to study the function of the apical and basolateral surfaces separately. (a) MDCK cells were cultured in dishes coated with a thick layer of collagen gel on which they form a monolayer (Misfeldt et al., 1976). After reaching confluence, the collagen sheet was detached from the dish and maintained floating in the culture medium. When the gel stayed attached to the culture dish, the component in the culture medium had direct access only to the apical surface of the cell layer, whereas the accessibility to the basolateral surface was restored with floating collagen sheet. In this system, however, the apical and basolateral surfaces are unable to be separated from each other. (b) Cells were cultured on a membrane filter attached to the bottom of a plastic tube. When the cells became confluent, the apical and basolateral surfaces were able to be separated by the cell layer. With this experimental system the apical and basolateral surfaces of the cell layer were accessible separately.

We report here the cAMP production of MDCK cell layer in response to AVP when this peptide had access to the apical and basolateral surfaces separately.

Materials and Methods

Cell Culture

The MCDK cells were purchased from Dai-nippon Pharmaceutical Co. Ltd. (Suta, Japan). The cells were plated at $4 \times 10^3$ cells/cm² and cultured in a 1:1 mixture of Dulbecco's modified MEM (DMEM) and Hams' F12 supplemented with 10% fetal calf serum (FCS) (GIBCO) at 37°C in a humidified atmosphere of 5% CO₂ in air. The medium was changed every 3 days and subculture was carried out every week.

The experiments were preformed by means of the following two systems.

(a) Culture on collagen gel sheet. Sterile hydrated collagen solution for tissue culture was purchased from Wako Junyaku Co. Ltd. (Osaka). Twenty four milliliters of collagen solution was mixed with 6 ml of a sterile buffer solution (0.1 M Na₂HPO₄, 0.65 M NaCl in distilled water). Then each well of the culture plates (9.6 cm²/well, Falcon) was coated with 500 µl of collagen solution quickly. The plates were kept in the incubator at 37°C for 3 hours. Each well was washed with Hanks' balanced salt solution twice before inoculation of the cells. Three milliliters of the cell suspension ($5 \times 10^5$ cells) was plated in a well. Within 4 days, the cells usually grew to form a confluent monolayer covering the collagen sheet. Then, the gel supporting the monolayer was detached from the dish by rimming at the edge of the gel prior to preincubation. AVP, added to the culture medium, was in contact with the apical cell surface when the gel was attached to the dish, and with both the apical and basolateral surfaces when the gel was floating.

(b) Culture on the membrane filter. Two milliliters of the cell suspension ($2 \times 10^5$ cells/ml) was seeded in a chamber equipped with a Millipore filter (HAWP 02500) glued to a plastic tube (13 mm in diameter), where the filter formed a permeable bottom to the chamber. The chamber was placed in a 50 ml centrifuge tube containing 2 ml culture medium. The bottom of the centrifuge tube was conical, allowing free diffusion of the medium through the filter (Figure 1). AVP was in contact either with the apical surface of the cell layer when added inside the chamber, or with the basolateral surface when added into the medium outside the chamber.

Hormone Preparations

Arginine vasopressin (AVP) was purchased from Protein Research Foundation (Minoh, Japan) and used after being dissolved in 0.001 N HCl containing 0.2% bovine serum albumin (Fraction V, Sigma).

Measurements of Transepithelial Potential Difference

A bridge of 3.0 M KCl/3% agar in teflon tubing, connected to a calomel half-cell was in-
Fig. 1. Millipore filter chamber and 50ml centrifuge tube. The filter chamber was placed in the centrifuge tube when the cells were cultured. Note the Millipore filter (arrows) glued to a plastic tube.

Introduced into the solution on each side of the filter and potential differences were measured with a differential electrometer (W.P. Instruments, Model F223A).

**Cyclic AMP Levels after Hormone Stimulation**

(a) After reaching a confluent monolayer, which was confirmed by using the phase contrast microscope, the culture medium (a 1:1 mixture of DMEM and Hams' F12 supplemented with 10% FCS) was replaced with the experimental medium (1 ml of a 1:1 mixture of DMEM and Hams' F12 containing 1 mM iso-butyl-methyl-xanthine). Immediately after replacement of the medium, the gel supporting the monolayer was detached from the plastic dish. Hormone solution was added to the medium after the 60 min preincubation at 37°C and another 10 min incubation was performed on the Dubnoff metabolic shaker. Twelve percent trichloroacetic acid (1 ml) was added to each well (final, 6%) to terminate the reaction. Cells were scraped and combined cells and media were transferred to the tubes. After freezing and thawing them six times, they were centrifuged at 3,000 rpm for 20 min. The supernatant solution was washed three times with diethyl-ether to remove trichloroacetic acid. After evaporating diethyl-ether from the supernatant solution in the vacuum drying oven for 6 hours at 20°C, the cAMP content was measured by radioimmunoassay according to the method of Steiner et al. (Steiner et al., 1969) using the antiserum kindly provided by K. Martin (Martin et al., 1978).

When the time course effects of AVP on the intra- and extracellular cAMP contents were studied, medium was transferred to ice-cooled tubes and 1 ml of 6% trichloroacetic acid was added to each well after an appropriate incubation period as indicated in the “Results.”

(b) Confluence of the monolayered cells on the filter was confirmed by the following three methods. (1) The microscopic examination of the filter after staining (Figure 2 a, b). (2) 24-hour constancy of the fluid-level difference between the medium in the filter chamber and that in the outer centrifuge tube after replacement of the medium (Figure 3). Thirty millimeter difference in the fluid-level was made at the time of the medium replacement. Only the cell layers which had maintained this 30 millimeter fluid-level difference for 24 hours were employed. (3) Development of a potential difference between the apical and basolateral surface of the cell layer. When the cells reached confluence, the culture medium was replaced with the experimental medium in the filter chamber (0.5 ml) and in the outer centrifuge tube (1.5 ml). The cells were preincubated on the Dubnoff metabolic shaker at 37°C for 60 min and then the hormone solution or solvent alone as a control was added into the medium in the filter chamber or the medium in the outer centrifuge tube. After a 10 min incubation period, all the experimental medium was removed and 1 ml of 6% trichloroacetic acid was added to the cell layer to stop the reaction. After the extraction of cAMP from the cells, its contents were measured.

**Results**

**Experiment (a)**

As shown in Figure 4, a dose-dependent increase in cAMP production was observed at a concentrations of AVP from one to
Fig. 2a, b. MDCK cells cultured on the Millipore filter were stained with Hematoxylin and Eosin. OPTIPHOT XF microscope (Nikon, Tokyo) was used to confirm the confluence of the cells. In the confluent state, the surface of the filter was covered completely with MDCK cells (a) (×200). When the cells did not reach confluency, spaces not covered with cells (arrow) were found (b) (×200).
Fig. 3. Difference in the fluid-level (arrows) between the medium in the filter chamber and that in the outer centrifuge tube. If the cells grow to reach a confluent state (left), the difference in the fluid-level is maintained for more than 24 hours after the addition of the medium in the filter chamber. If the cells do not reach a confluent state (right), the difference in the fluid-level is not maintained.

Fig. 4. Dose-dependent effect of arginine vasopressin (AVP) on cAMP production by MDCK cells cultured on collagen sheet. The increase in cAMP production was more prominent when the collagen sheet was floating (○—○) than when the sheet was kept attached (●—●). Values are expressed as Mean±S.E. for three different plates.

* P<0.01 significantly different between the attached and floating collagen sheet.

100 ng/ml when the hormone was added to the cells cultured on the attached collagen gel. An increase in cAMP production was not observed with the addition of AVP at 0.1 ng/ml. In contrast, when the gel supporting the monolayer was released before adding AVP, a dose-dependent increase in cAMP production was observed at a concentration of AVP from 0.1 to 100 ng/ml. The increase in cAMP production from the basal level was significantly higher in the cells on the floating gel than that on the attached gel. For example, when incubated with 100 ng/ml AVP, the increase in cAMP production of the cells on the floating gel (280 pmol/well) was 5 times greater than that on the attached gel (50 pmol/well). Thus, the increase in cAMP production was exaggerated when AVP was accessible to the basolateral surface.

Figure 5 shows the increase in intracellular and extracellular (medium) cAMP in response to AVP as a function of time when AVP was added to the cells on the attached collagen gel. The increase in intracellular cAMP reached its maximum within 2 min and the cAMP content remained on the plateau for another 60 min. On the other hand, the cAMP content in the medium was less than 1.25 pmol/9.6 cm² plate.
Experiment (b)

At the time of cell confluency on the filter, the potential difference between the apical and the basolateral surfaces of the cell layer developed and was $0.98 \pm 0.15$ mV (Mean ± S.E., n=6), with the apical surface negative. At this stage, a dose-dependent increase in cellular cAMP content was observed when AVP was added in the medium in the filter chamber or that in the outer centrifuge tube (Figure 6). The maximal increase in cAMP content was observed at a concentration of $100 \text{ng/ml}$ of AVP. The cAMP content in the monolayer cells stimulated on the basolateral surface by the various concentrations of AVP was significantly higher than that in the cells stimulated on the apical surface. The maximal increase in cellular cAMP content was 11 pmol/well when basolaterally stimulated. On the other hand, the maximal increase in cAMP content was only 3.5 pmol/well when apically stimulated.

The results of experiments (a) and (b) showed that the AVP responsive cAMP production systems may be present predominantly on the basolateral surface of the cultured MDCK cells.

Discussion

We have reported here that AVP increased the cAMP content of the monolayers formed by MDCK cells, particularly when AVP was accessible to the basolateral surface of the monolayer cells. Therefore it appears that the AVP-responsive cAMP production system may predominantly be located on the basolateral surface of the cultured MDCK cells.

MDCK is a cell line derived from dog kidney (Gaush et al., 1966) with many characteristics corresponding to collecting tubules such as positive responsiveness to AVP (Rindler et al., 1979). MDCK cells, grown in culture, form monolayers and exhibit a morphological polarization, suggesting that the upward-facing membrane corresponds to the luminal membrane of the epithelial cells and the membrane facing the culture dish surface corresponds to the basolateral
membrane (Leighton et al., 1970; Cereijido et al., 1978; Khayim et al., 1981). Furthermore, the functional polarity of the monolayers was confirmed by the transportation of water from the apical surface to the basolateral surface and the development of transepithelial potential of the cells cultured on the membrane filter.

Our experiments disclosed the MDCK cells increased their cAMP content, predominantly when AVP was accessible to the basolateral surface of the monolayer. Similarly, Garcia-Perez et al. (1984) reported that an increase in cAMP production was observed only when AVP was added in the basolateral medium of the monolayer formed by canine cortical collecting tubule cells obtained by immuno-dissection. Asymmetry of the apico-basolateral membrane might be explained by the following: First, the difference in the number of AVP receptors. The second possibility is the difference in the affinity of receptors to AVP molecules. Thirdly, the difference in the coupling of AVP receptors to adenylate cyclase moieties. Another explanation is the difference in the availability of ATP (substrate of adenylate cyclase).

The monolayers reconstructed by MDCK cells minimally increased cAMP production even in response to apical AVP administration. The reason for the minimal responsiveness to apical AVP stimulation could be the presence of a minimal number of AVP-responsive cAMP production systems on the apical membrane or the accessibility of AVP to the basolateral side by diffusion through the monolayer from the the apical side.

Although hormones have been usually added to the culture medium to study the biological action on the cultured renal epithelial cells, it seems important to take into consideration the accessibility of the hormone to the basolateral surface in the study of hormone action on the renal tubular cells.

References


