

Electrical Currents Flow Out of Domes Formed by Cultured Epithelial Cells

KAZUHIRO SUGAHARA,* JOHN H. CALDWELL,† and ROBERT J. MASON*

*Department of Medicine, National Jewish Hospital and Research Center, and †Department of Molecular and Cell Biology, National Jewish Hospital and Research Center, and Department of Physiology, University of Colorado Medical School, Denver, Colorado 80206

ABSTRACT Domes are localized areas of fluid accumulation between a cultured epithelial cell monolayer and the impermeable substratum on which the cells are cultured in vitro. Dome formation has been documented in a variety of epithelial cell lines that retain their transepithelial transport properties in vitro. However, it is not known whether domes are predominantly areas of specific active transport, or, alternatively, are predominantly areas of relative weak attachment to the culture surface. In the present study we adapted a vibrating microelectrode, which can detect small currents flowing in extracellular fluid, to determine if current was flowing into or out of domes and thereby to determine if domes were specialized areas of active transport. We used alveolar type II cells as the main epithelial cell type because they readily form domes in vitro and because they transport sodium from the apical to the basal surface.

We found that electrical current flowed out of domes. The direction of the current was independent of the size of a dome, of the age of an individual dome, and of the number of days in primary culture for alveolar epithelial cells. This current was inhibited by amiloride and ouabain and was dependent on sodium in the medium. We made similar observations (outward current from domes which is blocked by amiloride and by sodium substitution) with domes formed by the Madin-Darby canine kidney cell line. The data support the hypothesis that sodium is transported across the entire monolayer and leaks back mainly through the domes. We conclude that domes in epithelial monolayers are not predominantly special sites of active transport but are more likely simply areas of weak attachment to the substratum.

Domes are focal regions of fluid accumulation between a cultured epithelial cell monolayer and the impermeable substrata on which the cells are cultured in vitro (11). A variety of epithelial cell lines, which retain their transepithelial transport properties in vitro, form domes in culture (5, 9). It has been recognized that an impermeable substratum, an effective permeability barrier provided by tight junctions, and net unidirectional ion transport provided by morphologically polarized cells, are all essential for dome formation in vitro (5, 15, 18, 19). Since it has been difficult to study epithelial transport in cultured cells and easy to estimate the number and size of domes, many investigators have used dome formation as a quantitative estimate of active transport in cultured epithelial cells (7, 15, 21). However, it is not known whether domes are specific sites of active transport or, alternatively, are areas of weak attachment to the culture surface. In the present study, we used the vibrating microelectrode to measure current density around domes. The vibrating probe has been used to measure steady ionic currents in extracellular fluid of a wide variety of biological systems (17). However,

the technique has rarely been applied to cultured epithelial cells.

We have previously reported that alveolar type II cells form a polarized epithelial monolayer in culture with microvilli at their apical surface, tight junctions between cells, and a basal lamina beneath the cells, and that these cells actively transport sodium from the apical to basal surface as shown by formation of domes and by their bioelectrical properties in a Ussing chamber (14). In the present study we adapted a vibrating microelectrode to determine if current was flowing into or out of domes, which allows us to clarify the electrophysiological mechanism by which domes are formed in cultured epithelial cells. A preliminary report of some of this work has been presented (20).

MATERIALS AND METHODS

Cell Isolation and Culture: Alveolar type II cells were isolated from adult rat lung dissociated with elastase and were then partially purified on a discontinuous metrizamide density gradient (8). The cells were suspended at

10⁶ cells/ml in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 µg/ml gentamicin, 100 U/ml penicillin, and 50 µg/ml streptomycin. After type II cells had adhered overnight, the monolayers were rinsed with medium and the nonadherent cells were discarded. The resultant adherent cells, whether cultured on plastic dishes or on dishes coated with extracellular matrix made by bovine corneal endothelial cells (8), were >90% type II cells, as judged by the modified Papanicolaou's stain (6).

Vibrating Microelectrode Technique: The vibrating microelectrode technique was used as described previously (2, 10). In brief, the vibrating microelectrode is a metal-filled glass micropipette with an electroplated platinum ball (10–15 µm diam) on its tip and is attached to a piezoelectric bender, which is oscillated at a constant frequency (350 Hz in these experiments). Excursion distance of the electrode tip is ~30 µm. The input signal is led to a lock-in amplifier (Princeton Applied Research, Princeton, NJ) which is a phase-sensitive detector. The output of the amplifier provides a measure of the peak-to-peak potential difference between the electrode tip at its excursion limits. Each electrode is calibrated with a known current source. A potential difference of 50 nV (peak-to-peak) can be readily detected. The resistivity of the balanced salt solution is 75 ohm·cm; thus, 50 nV corresponds to a current density of ~0.25 µA cm⁻².

Experimental Procedure: After 2 or 3 d in culture, alveolar type II cells maintained directly on plastic culture dishes formed small domes (~100–400 µm diam), and type II cells maintained on the extracellular matrix adhered better and formed larger domes (~800–1,200 µm diam). After 2 to 7 d in culture, cells were washed and placed in warmed balanced salt solution (30–35°C), which is composed of 137 mM NaCl, 5.4 mM KCl, 0.6 mM MgSO₄, 1.1 mM CaCl₂, 1.08 mM Na₂HPO₄, 1.1 mM KH₂PO₄, and 5.5 mM glucose (pH 7.4). The movement of the vibrating microelectrode was oriented parallel to the monolayer and approximately perpendicular to the side of the dome. The vibrating microelectrode was placed at the edges of a dome and at a height midway between the top of a dome and the flat monolayer. For technical reasons the microelectrode could not be placed directly perpendicular to the top of a dome or to the flat portion of the monolayer.

Materials: Choline chloride was purchased from GIBCO (Grand Island Biological Company, Grand Island, NY), and 4-acetamide-4'-isothiocyano-2,2'-disulfonic stilbene was purchased from ICN Pharmaceutical, Inc. (Cleveland, OH). Amiloride was the kind gift of Merck Sharp & Dohme Research Laboratories, Division of Merck & Co., Inc. (Rahway, NJ). Reagents for culture were obtained as reported (6, 14).

RESULTS AND DISCUSSION

We found that current (by convention the movement of positive charge) flows out of domes made by alveolar type II epithelial cells and Madin-Darby canine kidney cells. This outward current was observed with all domes, independent of the number of days in culture, of the size of a dome, and of the age of an individual dome. The magnitude of the current was 2.3 ± 1.2 µA/cm² (mean ± SD, *n* = 15 domes in seven experiments) on plastic dishes, and 28.5 ± 10.2 µA/cm² (*n* = 16 domes in seven experiments) on extracellular matrix. The large currents were usually but not always found in larger domes. The outward current from a dome was not symmetrical; that is, it often was much larger from one side of a dome (*n* = 6, Fig. 1). There are several possible reasons that the currents are larger when cells are grown on extracellular matrix: (a) tight junctions between cells in the flat part of the monolayer might be tighter; (b) the domes could be more leaky, or (c) the subepithelial space with the matrix might be less resistive to current flow due to a greater separation between the monolayer and the dish.

One possibility for the outward current from a dome might be due to the mechanical damage by the vibrating microelectrode. However, we believe that the current is not due to damage and thereby imminent collapse of a dome for the following reasons: (a) the currents from individual domes have been measured for many hours; (b) under direct observation after current measurement, domes remained stable and lasted from 30 min to more than 7 h; and (c) when we deliberately hit a dome with the vibrating probe, the dome

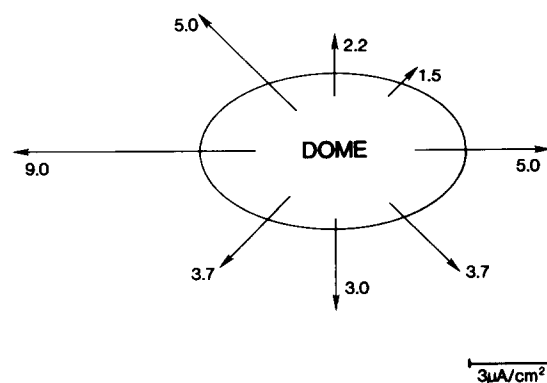


FIGURE 1 Current density around a dome. Current measurements are at the center of vibration of the probe (~25–30 µm from the membrane). The outward currents were asymmetrical around a dome. The larger current was not always associated with the portion of the dome with the smallest radius of curvature.

collapsed rapidly, and as the dome collapsed, the current increased markedly and even persisted after the dome collapsed.

Since dome formation is thought to be an indicator of active transport, we investigated the effects of inhibitors of transport. Fig. 2A shows the effect of amiloride, an inhibitor of apical sodium channels (1), on the current out of domes. Amiloride rapidly inhibited the current (*n* = 9). At a concentration of 10⁻⁶ M, it inhibited the current by >50% of initial value, and 10⁻⁴ M amiloride almost completely eliminated the current. Half-maximal inhibition was achieved with ~2 × 10⁻⁷ M amiloride (*n* = 3). These effects were readily reversible. Ouabain (10⁻⁴ M), an inhibitor of Na⁺-K⁺-ATPase, inhibited the outward current gradually and required 1 h for its full effect (80% inhibition, *n* = 3). The effects of ouabain were not reversible in 20 min. The slow time-course for ouabain effect is reasonable since ouabain blocks Na⁺ transport by inhibiting Na⁺-K⁺-ATPase on the basal surface, whereas amiloride acts immediately because amiloride blocks Na⁺ entry on the apical surface. Our results are consistent with the reports that the formation of domes is inhibited by amiloride and ouabain (7, 12, 13, 14). The inhibition of the current by amiloride and ouabain indicates that outward current from domes requires active sodium transport by the monolayer.

To study the importance of sodium for the outward current, we replaced all the sodium in the medium with choline, an impermeant cation. Fig. 2B shows the effects of sodium substitution on the outward current from domes. The low Na⁺ solution reversibly abolished the outward current within a few minutes. This effect was probably due to a gradual reduction of internal sodium concentration, so that the Na⁺-K⁺ pump was no longer active. In some cases the replacement of the Na⁺ led to a temporary reversal of the current before it disappeared. One possible explanation for this is that diffusion potentials for choline and sodium were established when the medium was changed (apical surface only) and the net diffusion potential across the paracellular pathway resulted in a transient reversal of current flow.

Substitutions of Cl⁻ by isethionate or K⁺ by Na⁺ had no significant effect on this outward current. Similarly, furosemide (10⁻⁴ M), an inhibitor of Cl⁻ transport (3), 4-acetamide-4'-isothiocyano-2,2'-disulfonic stilbene, an anion exchange inhibitor and Cl⁻ transport inhibitor (4), and BaCl₂ (50 µM),

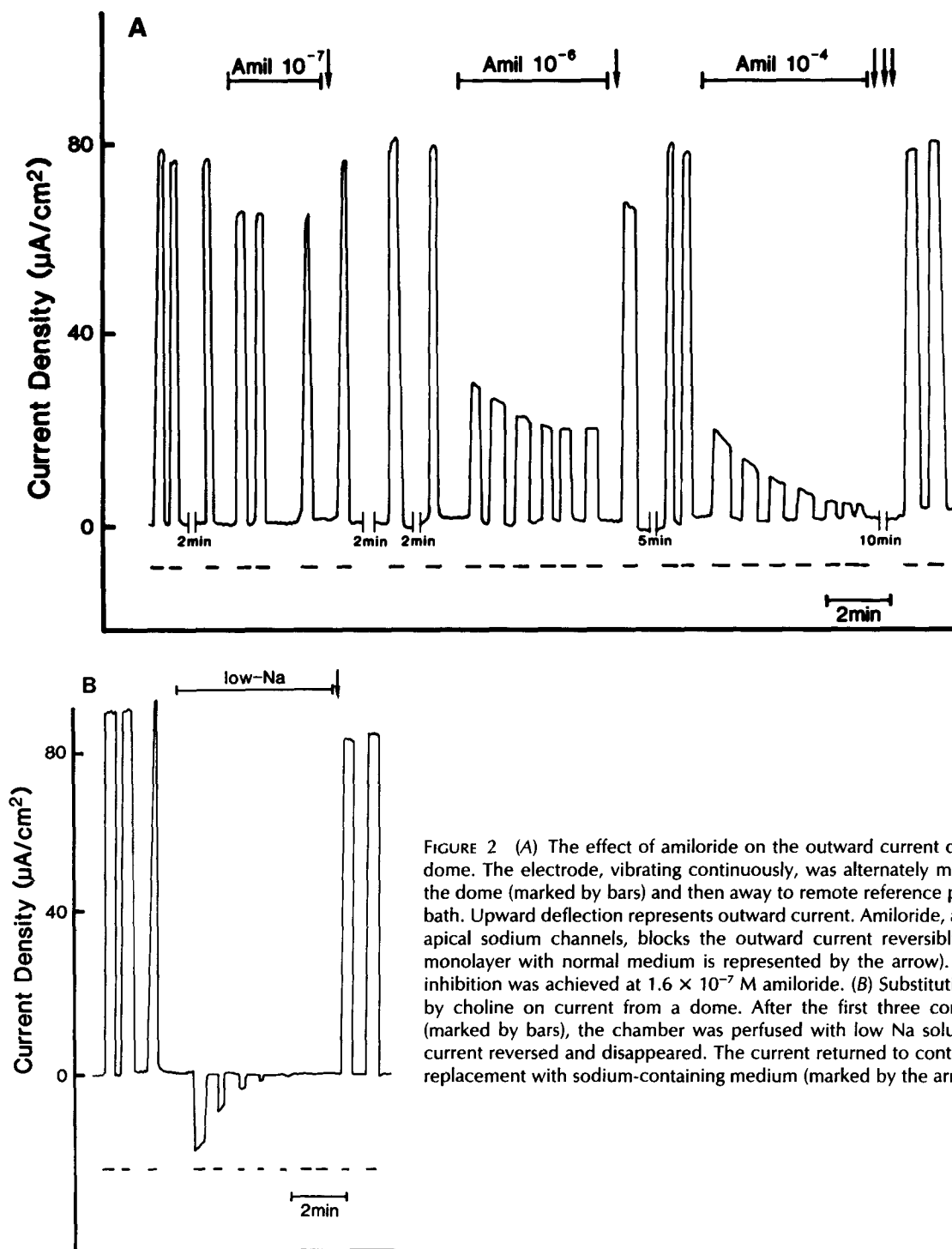


FIGURE 2 (A) The effect of amiloride on the outward current density from a dome. The electrode, vibrating continuously, was alternately moved close to the dome (marked by bars) and then away to remote reference position in the bath. Upward deflection represents outward current. Amiloride, an inhibitor of apical sodium channels, blocks the outward current reversibly (rinsing the monolayer with normal medium is represented by the arrow). Half-maximal inhibition was achieved at 1.6×10^{-7} M amiloride. (B) Substitution of sodium by choline on current from a dome. After the first three control readings (marked by bars), the chamber was perfused with low Na solution, and the current reversed and disappeared. The current returned to control value after replacement with sodium-containing medium (marked by the arrow).

a K^+ conductance inhibitor (16), had little effect on the outward current. Goodman et al. (7) recently reported that formation of domes by alveolar type II cells was not affected by substitution of Cl^- or K^+ . The findings reported in the present study suggest that the presence of Na^+ is essential for the outward current from domes formed by alveolar type II cells. We made similar observations (outward current from domes that is blocked by amiloride and by choline substitution for sodium) on domes formed by the Madin-Darby canine kidney cell line, and thus transepithelial sodium transport may be required for the outward current from domes by epithelial cells in general.

In the present study, we demonstrated that current flows out of domes formed by alveolar epithelial monolayers and that this current is dependent on extracellular sodium. It would be helpful to determine the current direction and density over the flat part of the epithelium, but it is technically very difficult. Our previous study (14) demonstrated that the alveolar epithelial cell monolayer cultured for 6–14 d on collagen-coated filters transports sodium actively from the apical side to basolateral side; the open circuit potential difference is ~ -1.0 mV, apical side negative, and the short circuit current is $\sim 2.0 \mu A \cdot cm^{-2}$. From the results presented here and in our previous study (14), we suggest the following

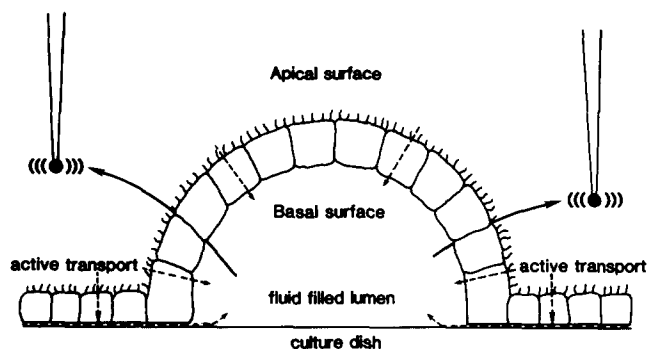


FIGURE 3 Schematic representation of the current from a dome. Alveolar type II cells actively transport sodium from the culture medium to beneath the monolayer (dashed arrows), which results in fluid accumulation between the cell monolayer and the culture dish, and sodium leaks back into the culture medium through domes (solid arrows). The apical cell surface (microvilli) is shown in contact with the medium; the basolateral surface is shown facing the culture dish. We measured the current by the electrode, which was vibrated parallel to the monolayer and approximately perpendicular to the side of the dome.

hypothesis (Fig. 3). The epithelium transports Na^+ and an accompanying anion across the entire monolayer and water follows passively. The solute and water create a hydrostatic pressure which form a dome at a site of weak attachment to the substratum. The junctions between cells in a dome are stretched, the transepithelial resistance decreased, and current flows out of the dome. Thus, the outward current in a dome would be generated by both the dome and flat part of the monolayer. These ionic currents are additive so that in a dome the outward leak is much greater than the inward current due to active transport. In a dome there is likely to be the same inward current as in the flat part of the epithelium, but this inward current is masked by the large outward current passing between the cells of a dome. This hypothesis also explains the observation that the current density around a dome is not symmetrical (Fig. 1), because the magnitude of the outward current from domes is likely to depend on the tightness of intercellular junctions between cells in different parts of a dome. We conclude that domes in epithelial monolayers are not special sites of active transport. From the present study, one speculation for dome formation is that domes arise from areas of weak attachment to the substratum, and that the number of domes will be determined by the attachment to the substratum and the conductance of the

junctional complexes (paracellular pathway) as well as the net transepithelial transport by the entire monolayer.

We thank Mrs. Kathy Shannon and Diane Nicholl for technical assistance and Ms. Sandy Subotnick for secretarial assistance.

This study was supported by a grant from the National Heart, Lung and Blood Institute, HL-29891, and the Lord and Taylor Laboratory of Lung Biochemistry of National Jewish Hospital and Research Center/National Asthma Center.

Received for publication 16 April 1984, and in revised form 19 June 1984.

REFERENCES

- Benos, D. J. 1982. Amiloride: a molecular probe of sodium transport in tissues and cells. *Am. J. Physiol.* 242:C131-C145.
- Betz, W. J., and J. H. Caldwell. 1984. Mapping electric currents around skeletal muscle with a vibrating probe. *J. Gen. Physiol.* 83:143-156.
- Burg, M., L. Stoner, J. Cardinal, and N. Green. 1973. Furosemide effect on isolated perfused tubules. *Am. J. Physiol.* 225:119-124.
- Cabantchik, Z. I., and A. Rothstein. 1972. The nature of the membrane sites controlling anion permeability of human red blood cells as determined by studies with disulfonic stilbene derivatives. *J. Membr. Biol.* 10:311-330.
- Cerejido, M., E. S. Robbins, W. J. Dolan, C. A. Rotunno, and D. D. Sabatini. 1978. Polarized monolayers formed by epithelial cells on a permeable and translucent support. *J. Cell Biol.* 77:853-880.
- Dobbs, L. G., E. F. Geppert, M. C. Williams, R. D. Greenleaf, and R. J. Mason. 1980. Metabolic properties and ultrastructure of alveolar type II cells isolated with elastase. *Biochim. Biophys. Acta.* 618:510-523.
- Goodman, B. E., R. S. Fleischer, and E. D. Crandall. 1983. Evidence for active Na^+ transport by cultured monolayers of pulmonary alveolar epithelial cells. *Am. J. Physiol.* 245:C78-C83.
- Gospodarowicz, D., and C. Ill. 1980. Extracellular matrix and the control of proliferation of vascular endothelial cells. *J. Clin. Invest.* 65:1351-1364.
- Handler, J. S., R. E. Steele, M. K. Sahib, J. B. Wade, A. S. Preston, N. L. Lawson, and J. P. Johnson. 1979. Toad urinary bladder epithelial cells in culture: maintenance of epithelial structure, sodium transport, and response to hormones. *Proc. Natl. Acad. Sci. USA.* 76:4151-4155.
- Jaffe, L. F., and R. Nuccitelli. 1974. An ultrasensitive vibrating probe for measuring steady extracellular currents. *J. Cell Biol.* 63:614-628.
- Leighton, J., Z. Brada, L. W. Estes, and G. Justh. 1969. Secretory activity and oncogenicity of a cell line (MDCK) derived from canine kidney. *Science (Wash. DC).* 163:472-473.
- Lever, J. E. 1979. Inducers of mammalian cell differentiation stimulate dome formation in a differentiated kidney epithelial cell line (MDCK). *Proc. Natl. Acad. Sci. USA.* 76:1323-1327.
- Lever, J. E. 1981. Regulation of dome formation in kidney epithelial cell cultures. *Ann. NY Acad. Sci.* 372:371-383.
- Mason, R. J., M. C. Williams, J. H. Widdicombe, M. J. Sanders, D. S. Misfeldt, and L. C. Berry, Jr. 1982. Transepithelial transport by pulmonary alveolar type II cells in primary culture. *Proc. Natl. Acad. Sci. USA.* 79:6033-6037.
- Misfeldt, D. S., S. J. Hamamoto, and D. R. Pitelka. 1976. Transepithelial transport in cell culture. *Proc. Natl. Acad. Sci. USA.* 73:1212-1216.
- Nagel, W. 1979. Inhibition of potassium conductance by barium in frog skin epithelium. *Biochim. Biophys. Acta.* 552:346-357.
- Nuccitelli, R. 1983. Transcellular ion currents: signals and effectors of cell polarity. *Mod. Cell Biol.* 2:451-481.
- Rabito, C. A., R. Tchao, J. Valentich, and J. Leighton. 1978. Distribution and characteristics of the occluding junctions in a monolayer of a cell line (MDCK) derived from canine kidney. *J. Membr. Biol.* 43:351-365.
- Rabito, C. A., R. Tchao, J. Valentich, and J. Leighton. 1980. Effect of cell-substratum interaction on hemicyst formation by MDCK cells. *In Vitro.* 16:461-468.
- Sugahara, K., J. H. Caldwell, and R. J. Mason. 1983. Electric currents flow out of domes formed by alveolar type II epithelial cells in primary culture. *J. Cell Biol.* 97:312a. (Abstr.)
- Valentich, J. D., R. Tchao, and J. Leighton. 1979. Hemicyst formation stimulated by cyclic AMP in dog kidney cell line MDCK. *J. Cell Physiol.* 100:291-304.