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Mechanisms of proton secretion by carbonic anhydrase-containing cells in turtle bladder

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Turtle Bladder 의 탄산탈수효소를 함유한 세포에 의한 Proton 분비기작

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抄 錄

Turtle bladder 上皮細胞의 輸送機作을 *in vitro* 에서 효과적으로 연구하기 위하여 Lucite chamber 한가운데 상피조직을 두고 電壓固定法을 적용하여 상피 세포층의 膜電位를 측정한 후 급속 凍結하고 투과 및 주사형 電子顯微鏡으로 탄산 탈수효소를 함유하는 세포의 표면막 특성을 分析하였다. 膀胱의 粘膜層은 두 타입의 탄산탈수효소를 함유한 세포가 특징적인데 丁端部와 基底部 세포막에서는 각기 다른 수송의 특성을 나타내고 있다. 즉 α 및 β형 탄산탈수효소가 풍부한 세포는 丁端細胞膜의 proton 펌프를 이용하여 H^+ 分泌에 관여하거나 bicarbonate의 再吸收 기능을 가지는 것으로 믿어진다. 본 연구에서 탄산탈수효소를 함유한 α형의 세포의 proton 分泌輸送과 세포막 투과성 변화와의 상관관계를 관찰하였는 바, 이들 세포에서 H^+을 분비하는 과정에서 정단부의 表面細胞膜 P-face에는 특이한 구조로서 細胞膜內 粒子들이 다량으로 분포하였다. 이와같은 細胞膜內 粒子들은 proton 펌프를 함유하는 것으로 생각되며 β형의 세포에서는 基底細胞膜에서 관찰되고 있다. 이와같은 결과는 膀胱上皮 세포내 탄산탈수효소는 H^+과 HCO_3^-의 생성에 관여하지만, 특히 α형 세포에서 정단세포막의 proton 펌프를 이용한 H^+ 分泌輸送과 기저세포막을 통한 bicarbonate의 再吸收 기능을 설명해 주는 중요한 사실로서 사료된다.

Key words: Turtle urinary bladder, Proton secretion, Freeze-fracture electron microscopy. α type of CA cell

INTRODUCTION

The cellular physiological study on the transport mechanisms of epithelial cell has been widely carried out centering in the collecting tubule system of mammalian kidneys, (Kaissling, 1982; Stanton, 1984; Brown and Orci, 1986; Brown *et al.*, 1988; Koeppen,

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1989; Stanton and Kaissling, 1989) amphibian skin (Nielsen, 1982) and urinary bladder cells (Cohen and Steinmetz, 1980; Steinmetz and Anderson, 1982; Gluck *et al.,* 1982; Fischer *et al.,* 1983; Durham and Matons, 1984; Sabatini, 1985; Clausen and Dixon, 1986; Steinmetz, 1986; Drenckhahn *et al.*, 1987; Jeon, 1989; Palmisano *et al.,* 1989), which plays an important role in studying a cellular organism.

The tissue of choice for studying urinary proton secretion is the turtle bladder because urinary epithelial tissue has a wider surface, so that it is effectively used for studying the mechanism of active transport in vitro. It is possible to measure the membrane potential of epithelial cell layer by using the voltage clamping technique after locating epithelial tissue in the center of a acrylic chambers and bathing on both sides by Ringer solutions with or without CO_2. For this reason, the studies have been actively carried out on the field of transport physiology for recent some years. In the previous article (Jeon, 1989), the ultrastructure of CA cells of the turtle bladder mucosa was presented by means of thin-section and freeze-fracture electron microscopy which is the best method for examining both function and membrane structure of transporting cells. In particular, the studies have been carried out on urinary bladder of turtle (Jeon, 1988, 1989, 1992) or toad (Jeon, 1990) as the typical model necessary to examine both function relation and structure of transporting membrane. In addition, physiological studies have been actively promoted by using the recently developed rapid freeze technique, as well as fine observation with traditional electron microscopy, while several models of transport mechanism have been suggested.

The turtle bladder epithelium has three cell types on mucosal surface and is believed to be responsible for a single transport mechanism (Steinmetz and Stetson, 1986). The recent description of three major transport mechanisms that occur in the turtle bladder are sodium reabsorption, proton secretion and bicarbonate secretion. It is believed that the majority (80%) of the mucosal cells are the granular cells. Husted *et al.,* (1981) found the remaining cells are rich in carbonic anhydrase. At present, the carbonic anhydrase-containing cell of turtle urinary bladder is divided into two types. Then, it seems that this type of cell takes part in H^+ secretion by using the proton pumps of apical cell membrane, or has a function to reabsorb bicarbonate. However. more studies are required for considering their regular transport pathways. Therefore, this study analysed the transport properties of urinary bladder cell known as the typical system of "tight epithelia" by using TEM observation with both freeze-fracture electron microscopy and thin-section method and mainly suggested the turtle bladder epithelial cell's H^+ transport function.

MATERIALS AND METHODS

Tissue preparation. Paired hemibladders from freshwater turtles, *Pseudemys scripta,* were removed and mounted in Lucite chambers having an exposed epithelial area of $8\,cm^2$. The hemibladders were bathed on both sides with a Ringer solutions containing in milimolar: 95 NaCl, 3.5 KCl, 2 Na_2HPO_4, 20 $NaHCO_3$, 1.8 $CaCl_2$ and 5 glucose. The both mucosal and serosal solution were bubbled with 5% CO_2 in air and had a pH of 7.2. The CO_2-free Ringer solution had the same composition except that the 20 mM $NaHCO_3$ were replaced by 10 mM Na_2SO_4 and 10 mM sucrose. This solution had a pH of 7.4 when bubbled with CO_2-free air. Ouabain (10^{-4} M) was added to the serosal solution on chambers and the reversed short-circuited current was then measured during approximately 2 hour periods on a potentiometric recorder. After the proton transport rates stabilized (approx. 2h), the hemibladders were fixed in the chambers with 2.5% glutaraldehyde in 0. 1 M cacodylate buffer (pH 7.4) and were prepared for thin-section and freeze-fracture transmission electron microscopy and for scanning electron micros-

copy.

Electron microscopy. For conventional thin-section electron microscopy of the surface epithelial cells, hemibladder tissues were then postfixed in cacodylate-buffered 1% OsO_4 for at least 2 hours, dehydrated in an alcohol series and embedded in a mixture of Epon. Thin sections were cut on glass knives and stained with urany acetate and lead citrate. In addition, some pieces of tissue were stained by the ruthenium red for examination of external surface of apical membrane in thin sections. Examination of thin section was carried out on a Hitachi H-500 electron microscope.

For freeze-fracture electron microscope, the glutaraldehyde fixed materials were soaked in 10% glycerol in 0.1 M cacodylate buffer for 2 hours and left in 25% glycerol in the same buffer overnight prior to placing on gold support stubs. After soaking in 25% glycerol to prevent ice crystal formation, bladder tissues were frozen at −150℃ in liquid Freon 22 cooled by LN_2. Freeze-fracture replicas were produced in a Balzers BAF 301 freeze-etch unit, equipped with an EVM 052 electron evaporation guns and a QSG quartz crystal thin-film monitor. Each frozen pieces of tissue was fractured at −110℃ for 10 minutes and a vaccum of 1×10^{-7} Torr and replicated with platinum and carbon from the electron-beam gun mounted at a 25° angle. Replicas were cleaned overnight in Chlorox, finally rinsed with several changes of distilled water and picked up on specimen grids. Tissue replicas were examined with a Zeiss EM 10CR electron microscope. The α type of CA cells were distinguished by means of freeze-fracture on the basis of distribution of rod-shaped intramembrane particles. In addition, frequency of cell types were determined from scanning electron micrographs.

For scanning electron microscopic observations, specimens were postfixed with cacodylate-buffered 1% OsO_4 for 2 hours, dehydrated by graded ethanol series at low temperature, frozed in liquid nitrogen at −196℃. They were then infiltrated with isoamyl acetate for 30 minutes and dried with critical point drier. The specimens were coated in 40 nm thickness with gold palladium and examined in an Hitachi S-450 scanning electron microscope.

Measurements of the short-circuit current. The rate of proton secretion was measured as the short-circuit current (I_{sc}) after sodium transport was inhibited with ouabain added to the serosal solution. The transepithelial voltage (V_T) of the mucous epithelium is oriented lumen negative and depends on the active reabsorption of Na^+, which is driven by ouabain-sensitive Na^+-K^+ ATPase located in the basolateral cell membrane. Inhibition of Na^+ transport, either by addition of amiloride to the luminal solution or by addition of ouabain to the serosal bathing solution abolishes the lumen negative V_T. Therefore, inhibition of Na^+ transport causes the V_T to reverse polarity, becoming lumen positive. This lumen-positive V_T appears to reflect active electrogenic H^+ secretion. When a stable I_{sc} was reached following ouabain addition, the bladders were fixed for transmission electron microscopy.

RESULTS

Cell types of the bladder mucosa.

The turtle urinary bladder, like other tight urinary epithelia, consists of a heterogenous population of cells as shown in Figure 1. The granular cell is found predominantly in the mucosa and may function primarily in the process of Na^+ transport in the turtle bladder. The remaining cells are the carbonic anhydrase (CA)-rich cells. When thin sections of bladder cells are examined, the granular cells characteristically have numerous apical mucin granules, a sparse number of mitochondria, luminal microvilli and no histochemically detectable CA activity. In contrast, the CA-rich cell is characterized by numerous mitochondria, a few small granules and histochemically demonstrable CA within the cytoplasm.

In addition, many electron-lucent vesicles are present in apical cytoplasm of α type of CA-rich cell as shown in Figure 1. It is believed that these vesicles probably contain proton pumps and may fuse to apical membrane.

As viewed in freeze-fracture electron micrograph (Fig. 5), the apical surface membranes of the α type of cells have a population of rod-shaped intramembrane particles, which are believed to be components of the proton pumps. It is of interest, however, that these particles are also observed in cytoplasmic membrane vesicles but are absent from the basolateral cell membrane. The β type of CA-rich cell, on the other hand, displays regular, rather short microvilli at luminal surface, as shown in Figure 2a. This type of cell shows array of membrane-limited tubules in apical cytoplasm. In addition, the apical surface area is small and mitochondria are widely distributed in the infranuclear cytoplasm. The epithelial cell in Figure 2,3 and 4 is a scanning electron micrograph of α type of CA cell which has a irregular microvilli or microplicae on their apical surface area.

Mechanisms of H^+ secretion by the CA-rich cells.

The cellular layer of urinary bladder cells plays the role as the barrier of transport materials, so that unlike single cell, they depend on a complex pathways of transport. Namely, they have particular properties of membrane transport which can not be explained by a simple model of transport pathway through cellular membranes. In turtle urinary bladder, they show individually different properties of transport in apical and basolateral plasma membranes, due to tight junction between epithelial cells, membrane permeability, shunt pathway and difference in ion compositions of both sides of apical cellular membrane.

The cellular morphology and physiological evidence suggests that the functional organization of the bladder epithelium can be represented by two

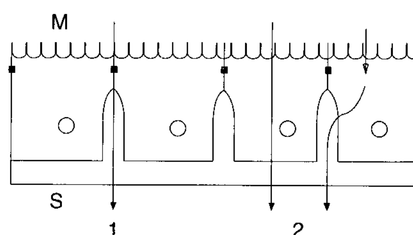

Fig. A. Schematic representation of two types of transepithelial pathways across the transporting epithelial cells. 1. Paracellular transport in between the cells. 2. Transcellular transport through the cells. M, mucosal surface; S, serosal surface.

transport pathways, one transcellular and the other paracellular. As illustrated in Figure A, the transcellular path includes the apical or luminal cell membrane, the cellular cytoplasm and the basolateral cell membrane. In contrast, the paracellular path is composed of the tight junction and the lateral intercellular space.

The comparison of turtle bladder's transport system in each cell type is diagrammed in Figure B. As shown in this Figure, two types of the CA-rich cells are responsible for the transport processes of H^+ and HCO_3^- secretion. Instead, the granular cells specialized for transcellular Na^+ transport and CA-rich cells are not directly involved in this process, implying that transport system of these cells are functionally separate and each is responsible for a single major transport system, namely, Na^+ reabsorption by granular cell, H^+ secretion by acid-secreting cell and HCO_3^- secretion by base-secreting cell. The α and β type of CA-rich cells in turtle bladder are both responsible for proton transport, but in opposite directions.

The transepithelial voltage (V_T) of the mucous epithelium is oriented lumen negative and depends on the active reabsorption of Na^+, which is driven by ouabain-sensitive Na^+-K^+ pumps located in the basolateral cell membrane. The conductive movement of Na^+ across the apical cell membrane is

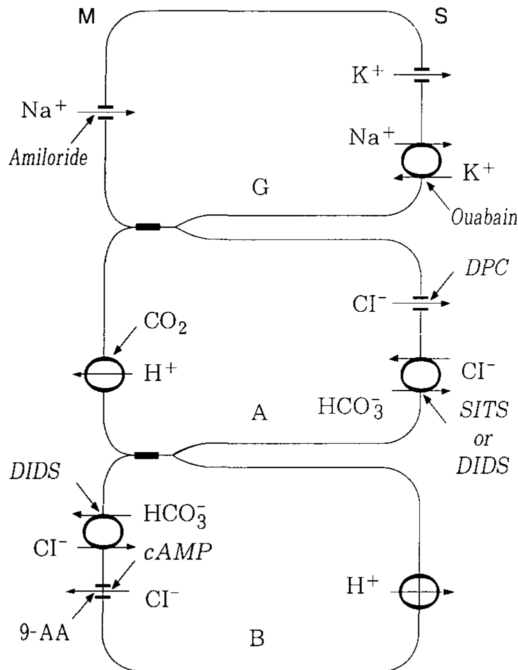

Fig. B. Schematic representation of transport systems of three major type of cells found in turtle bladder mucosa. Transport system of these cells are functionally separate and each is responsible for a single major transport system, namely, Na^+ reabsorption by granular cell (G), H^+ secretion by acid-secreting cell (A) and HCO^-_3 secretion by base-secreting cell (B). The transepithelial voltage (V_T) of the mucous epithelium is oriented lumen negative and depends on the active reabsorption of Na^+, which is driven by ouabain-sensitive Na^+–K^+ pumps located in the basolateral cell membrane. The α and β type of CA cells are both responsible for proton transport, but in opposite directions. The apical membrane is on left of cell (M) and the serosal surface is on right of cell (S).

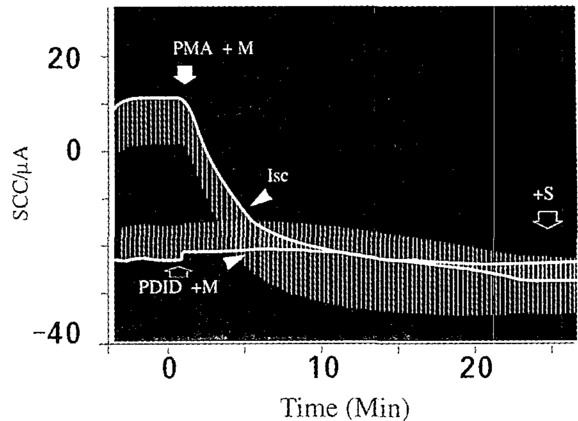

Fig. C. Short-circuit current traces of an experiment in which Na^+ absorption is inhibited by 0.5 mM ouabain in both experimental and control hemibladders. The varing concentrations of PMA and PDID were added to M sides (mucosal or apical) of both bladders at time 0 and the short-circuit current (Isc) was then measured for 30 minutes. Following another 30 minutes drug was added to S sides (serosal solution), as indicated by large clear arrow, to observe its changes on Isc response.

blocked specifically by amiloride. Inhibition of Na^+ transport, either by addition of amiloride to the luminal solution or by addition of ouabain to the serosal bathing solution abolishes the lumen negative V_T. Therefore, inhibition of Na^+ transport causes the V_T to reverse polarity, becoming lumen positive. This lumen-positive V_T appears to reflect active electrogenic H^+ secretion. As shown in Figure C, the short-circuit currents (SCC) were obtained with an experiment in which Na^+ absorption was inhibited by 0.5 mM ouabain to the serosal bathing solution with 25 mM bicarbonate-Ringer buffer and gassed with 5% CO_2 in a acrylic chambers. The addition of ouabain to S virtually abolished Na^+ transport resulting in reversed SCC (RSCC) and electrical potential difference (PD) across the bladder with S becoming negative with respect to M (Fig. A). This RSCC and reversed PD were stable for 0.5 h. Thus, this 0.5 h interval was designated at the control period.

DISCUSSION

The ultrastructure of the epithelium of the turtle urinary bladder has been described as a model system for examining the epithelial transport mechanisms by many investigators (Rosen, 1972; Schwartz

et al., 1982; Stetson and Steinmetz, 1983; Jeon, 1989). The urinary bladder has a simple histology, providing several square centimeters of hemibladder as a flat membrane between two bathing solutions. It is now clear that the bladder mucosa is consisted of three different cell types: a basal cell, a granular cell that accounts for about 80% of the surface epithelial cells and a third cell type, that is, carbonic anhydrase -rich (CA) cell population of the turtle urinary bladder. It is well known that the third cell type corresponds to the mitochondria-rich cell of the toad urinary bladder (Wade, 1976; Jeon, 1990).

The cytoplasmic membrane of epithelial cell is composed of an apical and a basolateral portion. As noted earlier, these two membranes of an epithelial cell have permeabilities and transport systems that have very different properties and give the cell a polarity. Thus, the functions of transport regulation are accomplished by polarized epithelial cells, that is, the surface membranes of the two opposite sides of the cell containing different transport systems. It has been proposed that the turtle bladder is a functional and morphological analogue of the mammalian cortical collecting tubules (Steinmetz, 1974), while the toad urinary bladder has a functional similarity in mammalian renal distal tubule. In turtle bladders, therefore, the principal pathways of transepithelial fluxes are believed to be confined to cells. Together with the tight junction, the adhering zonule and adhering macula form the so-called junctional complex which is characteristic of many epithelia. Preliminary reports of a bladder epithelial cell should also be noted, suggesting that the granular cells reabsorbs sodium and secretes potassium whereas the CA-rich cell is responsible for urinary acidification.

Previous studies by Steinmetz and Stetson (1986), have shown that exposure to CO_2 more than doubled the planar area of the apical surface of α type CA cells and increased the degree of amplification of the apical cell membrane. They also observed that the intramembrane particles (IMP) density appeared to be increased by about 40%. Therefore, the total CO_2 -induced increase in IMPs in position at the luminal surface was 5 fold while the increase in H^+ transport was 9-fold. They concluded that stimulation of H^+ transport by CO_2 involves recruitment of IMP to the apical cell membrane of α type CA cells and that IMPs are associated with active H^+ transport. As shown in scanning electron micrograph, it is clear that the apical surface area of α type CA cell exhibits marked changes when H^+ secretion by the bladder is stimulated by the addition of CO_2. In addition, thin-section and freeze-fracture electron micrograph indicated that its apical membrane is characterized by microplicae and contains numerous rod-shaped IMPs on freeze fracture.

Looking at the freeze-fracture electron micrograph, intramembranous particle was the only prominent structure in the apical membrane of the α type of CA cell. It is of interest, however. these rod -shaped particles do not appear on the apical membrane of the other type of CA cell when studied by freeze-fracture. Instead, it has been proposed that the β type of CA cell has a lower density of rod-shaped particles in their basolateral membranes, implying that α type of CA cell is responsible for acid transport using the proton pumps on the apical membrane, while the β type of cell secretes bicarbonate via an oppositely-directed proton pumps in their basolateral membrane. Moreover, a distinctive rod-shaped intramembrane particle has been described in freeze-fracture electron micrographs of plasma membrane from many cells thought to be responsible for acid transport (Humbert *et al.,* 1975; Wade, 1976; Brown. 1978; Stetson *et al.,* 1980; Jeon, 1990). Therefore, it is likely that there are several different proton pumps, one of which is responsible for urinary acidification and appears as rod-shaped particles in turtle urinary bladder cells.

One of the ways in which vertebrates control the pH balance of their blood is to excrete excess hydro-

gen ions. These hydrogen ions or protons are removed from the blood by cells in the excretory system and pumped into the urine. However, not all cells in the excretory system are able to transport protons in this manner. It is likely that only certain epithelial cells contain the proteins that makeup the proton pump mechanism. It has been reported that the transporting epithelial cells with proton pump activity can be found in the collecting ducts in mammalian kidneys as well as in bladders of the fresh water turtle, *Pseudemys scripta*.

It has been well known that the tight junctions, or also known as zonulae occludens, form a continuous seal around the granular epithelial cells which are the site of Na^+ transport systems in the bladder. In leaky epithelia, the tihgt junctions that join the cells are leaky to water, small molecules and ions, thus providing two types of pathways across the epithelia, as illustrated in Figure A: 1) transcellular transport through the cells and 2) paracellular transport in between the cells. However, it appears that the tight junctions constitute a tight seal restricting diffusion in tight epithelia such as mammalian urinary bladder, mammalian renal distal tubule and amphibian skin.

It is currently believed that in the absence of exogenous CO_2 and HCO_3^- the rate of H^+ transport is limited by the rate of metabolic CO_2 production and carbonic anhydrase catalyzed hydration of this CO_2. It has been described that the addition of low concentrations of CO_2 to serosal solutions (S) increases the rate of mucosal or apical-sides (M) acidification and, in contrast, high concentrations of CO_2 decrease acidification, probably due to S to M HCO_3^- flux, as previously demonstrated (Schwartz *et al.*, 1972, 1974; Steinmetz, 1974; Cohen and Steinmetz, 1980; Steinmetz and Stetson, 1986). Thus, it is apparent that the net rate of H^+ transport (J_H) cannot be determined by the rate of M acidification alone with added CO_2 and HCO_3^- to S because of the simultaneous transport of H^+ and HCO_3^- into M. In this study J_H was determined by the SCC applied across ouabain-treated bladders.

In terms of the Cl-HCO_3 exchanger, Drenckhahn *et al.*, (1987) have shown that both SITS and DIDS inhibit the basolateral exchanger by more than 80% in the α type of CA cells, whereas SITS fails to inhibit the apical exchanger of the β type of CA cell and DIDS inhibits it only slightly. Therefore, these and other studies (Drenckhahn *et al.*, 1987; Kohn *et al.*, 1989) suggest that the two Cl-HCO_3 exchangers of α and β type of CA cells in turtle bladder have different transport characteristics. Furthermore, although H^+ secretion and HCO_3^- secretion appear to represent physiologically separate processes, they are both inhibited by acetazolamide and independent of sodium transport (Stetson *et al.*, 1985). It is believed that these characteristics of the apical transporter remain to be clarified in turtle bladder epithelia.

ABSTRACT

This study was carried out to examine the H^+ transport mechanism by observing the properties of cellular membrane having an α type of carbónic anhydrase (CA)-containing cells in turtle urinary bladder. The urinary bladder consists of a heterogenous population of cells. As a result of fine observation with traditional thin-section electron microscopy, the bladder epithelium has three different cell types on mucosal surface. They are a basal cell, a granular cell and a third type of CA-rich cell. The CA-rich cells are divided into two distinct smaller groups within them and called them α type and β type of CA cells. The α type of CA cells are responsible for the proton secretion using the proton pumps on the apical plasma membrane, while the β type of CA cells secrete bicarbonate via an oppositely-directed proton pumps in their basolateral plasma membrane. After performing the freeze-fracture technique, it was shown that there were distributed a

large number of intramembranous particles having a special structure on the apical membrane of α type of CA-rich cells in the process of their H^+ secretion. In turtle bladder α type of CA-rich cells, this particle was the only prominent structure in the apical membrane. These intramembrane rod-shaped particles probably represent the integral membrane components of the proton pump. This result may explain that carbonic anhydrase within epithelial cell of urinary bladder takes part in formation of H^+ and bicarbonate, that active transport of H^+ is done, and that the reabsorption of bicarbonate suggests transport mechanism containing H^+ secretion. However, it seems that more studies are required for considering their regular transport pathway.

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ABBREVIATIONS

A	α type of CA-rich cell, acid-secreting cell
B	β type of CA-rich cell, bicarbonate -secreting cell
CA	carbonic anhydrase
Cy	cytoplasm
EF	outer half of plasma membrane
G	a granular cell
g	electron-dense mucous granules
M	mucosal or apical surface sides
m	mitochondria
PD	potential difference
PF	inner half of plasma membrane
RSCC	reversed short-circuit current
S	serosal surface sides
SCC, I_{SC}	short-circuit current
TEM	transmission electron microscope
TJ	tight junction
V_T	transepithelial voltage
cAMP	stimulates chloride secretion on α CA -cell
PDID	phorbol 12, 13-didecanoate

PMA	phorbol 12-myristate, 13-acetate	DIDS	4.4′-diisothiocyanostilbene -2.2′-disulfonic acid
SITS	4-acetamido-4′-isothiocyan ostilbene -2, 2′-disulfonic acid		

FIGURE LEGENDS

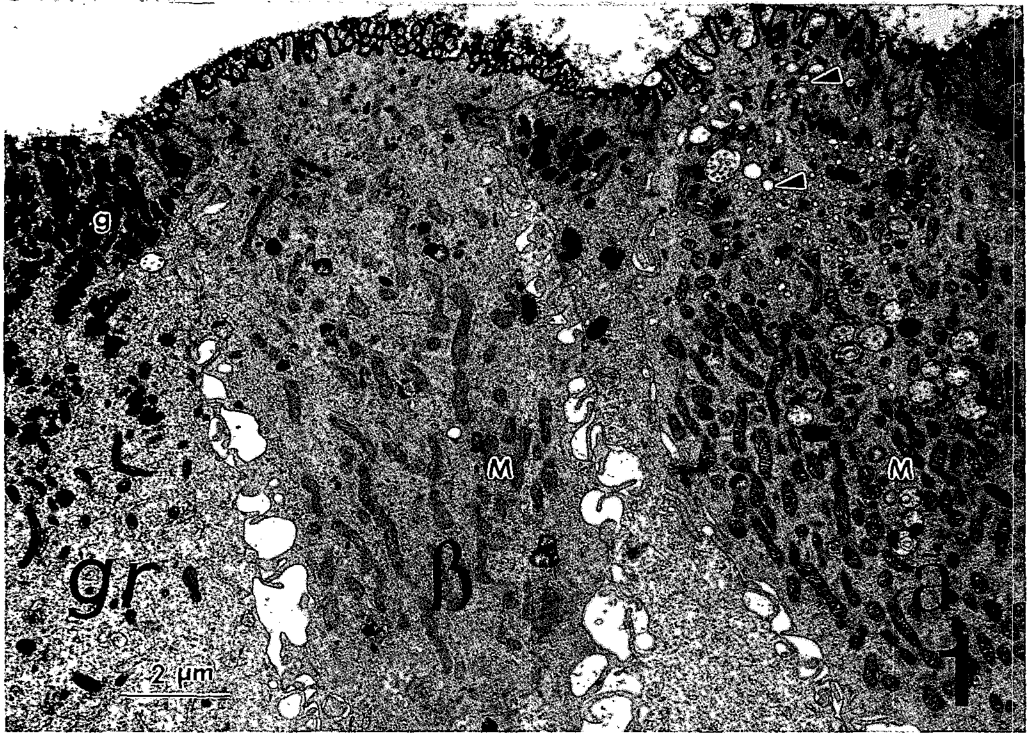
Fig. 1. Electron micrograph of the turtle urinary bladder. This tissue was stained with ruthenium red, a stain that binds to mucosubstances associated with external surface of apical membrane. The bladder mucosa has three main cell types on their mucosal surface: the granular cell (gr), the α type of CA-rich cell (α) and β type of CA-rich cell (β).Note the α type of CA-rich cell, showing many electronlucent vesicles (arrowheads) in apical cytoplasm. The apical membrane area of this cell reveals irregular projections and there are abundant mitochondria (M) in supranuclear cytoplasm. g, electron-dense mucous granules.

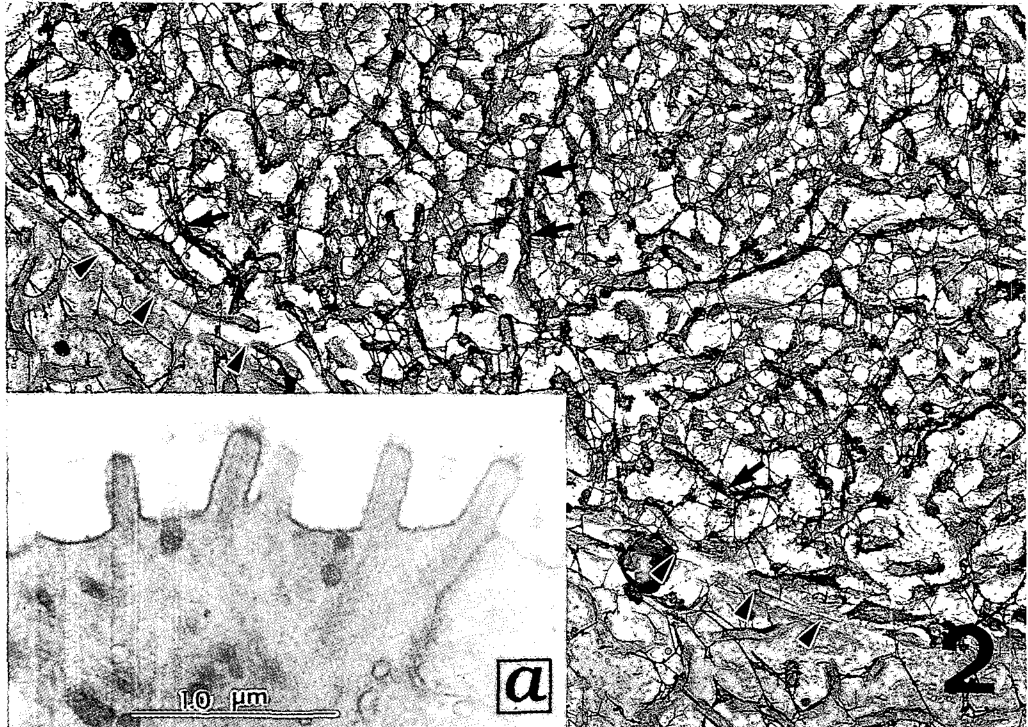
Fig. 2. Electron micrograph of luminal surface area by surface replica of turtle bladder epithelia, showing the granular cell with microvilli and the α type of CA-rich cell with irregular microvilli or probably with microplicae (arrows). The luminal surfaces of bladder cells are covered by a glycoprotein-rich surface coat. Also seen are the portions of boundary between two adjacent cells (arrowheads). a, β-CA cell.

Fig. 3. Scanning electron micrograph showing a surface view with prominent apical membrane microvilli characteristic of the α type of CA-rich cell. This tissue was fixed after exposure to 5% CO_2 and 20 mM HCO_3^- ringer's solution for 1h. Note that this cell occupies greater area and apical membrane is folded into an intricate network of microplicae (arrowheads).

Fig. 4. Stereomicrograph of a surface area prepared for microscopy by a technique called freeze-drying surface replica. Stereomicrograph pairs provide a three-dimentional image of cellular structures. This electron micrograph shows the white filamentous glycoprotein-rich surface coat. Also seen are the apical microplicated surface membrane, which are characteristic of the carbonic anhydrase-containing cells.

Fig. 5. Freeze-fracture electron micrograph of the apical surface membrane of α type of CA cell, showing a population of rod-shaped intramembrane particles (arrowheads), which are believed to be components of the proton pumps. The tight junction (TJ) is characterized by interweaving strands on the P-face and grooves on the E-face. Cy indicates cytoplasm of the α type of CA cell.


g
M
M
gr
β
α
2 μm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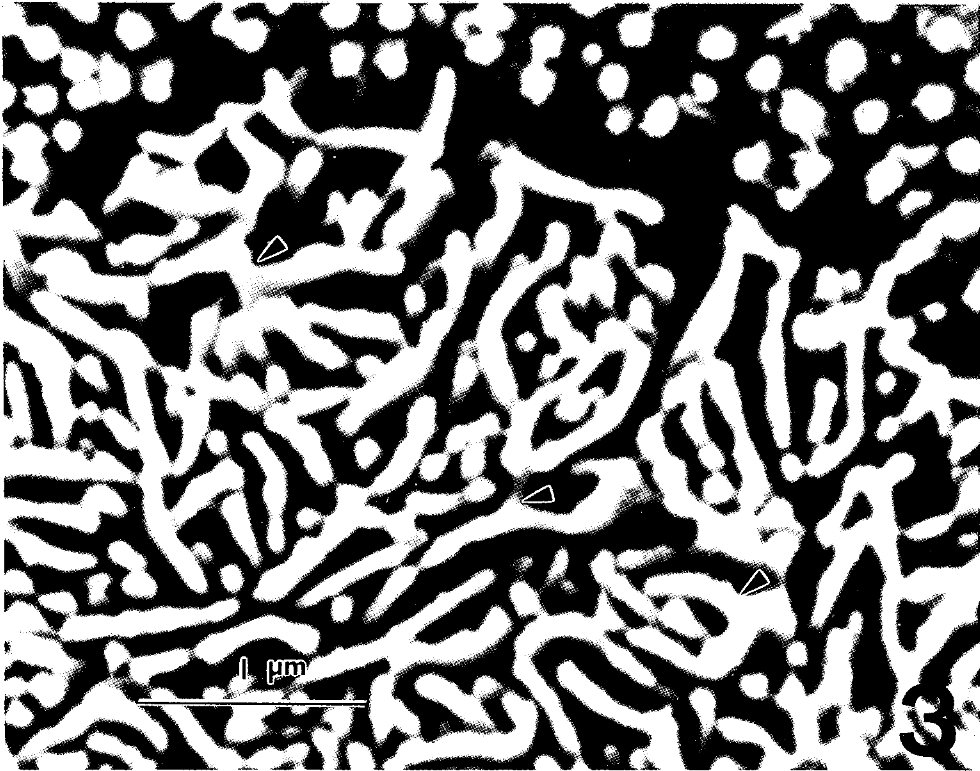
a
2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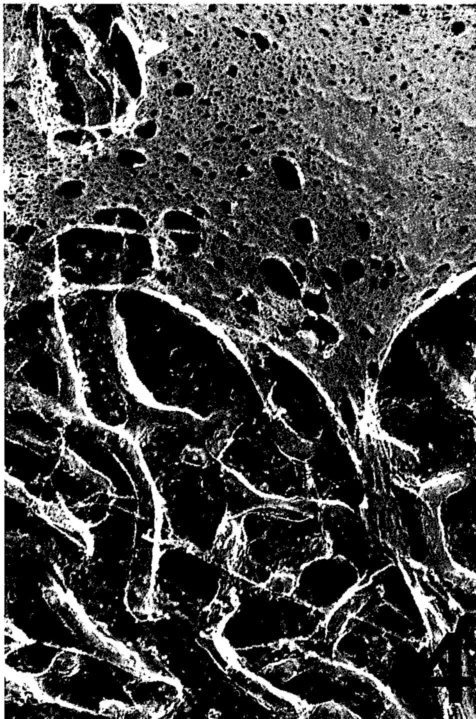
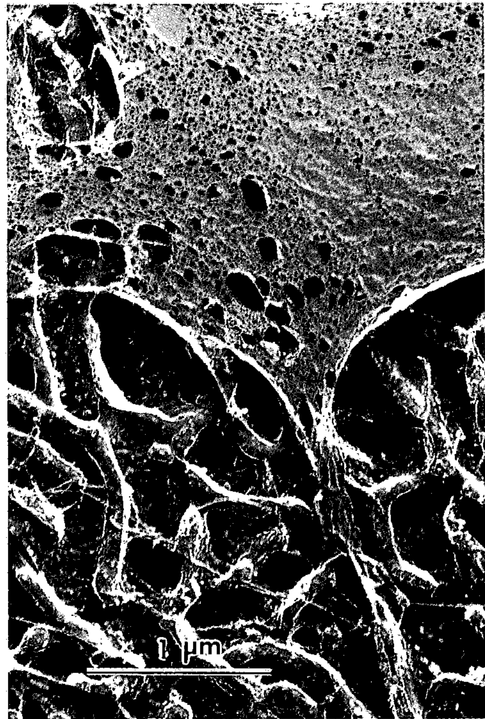
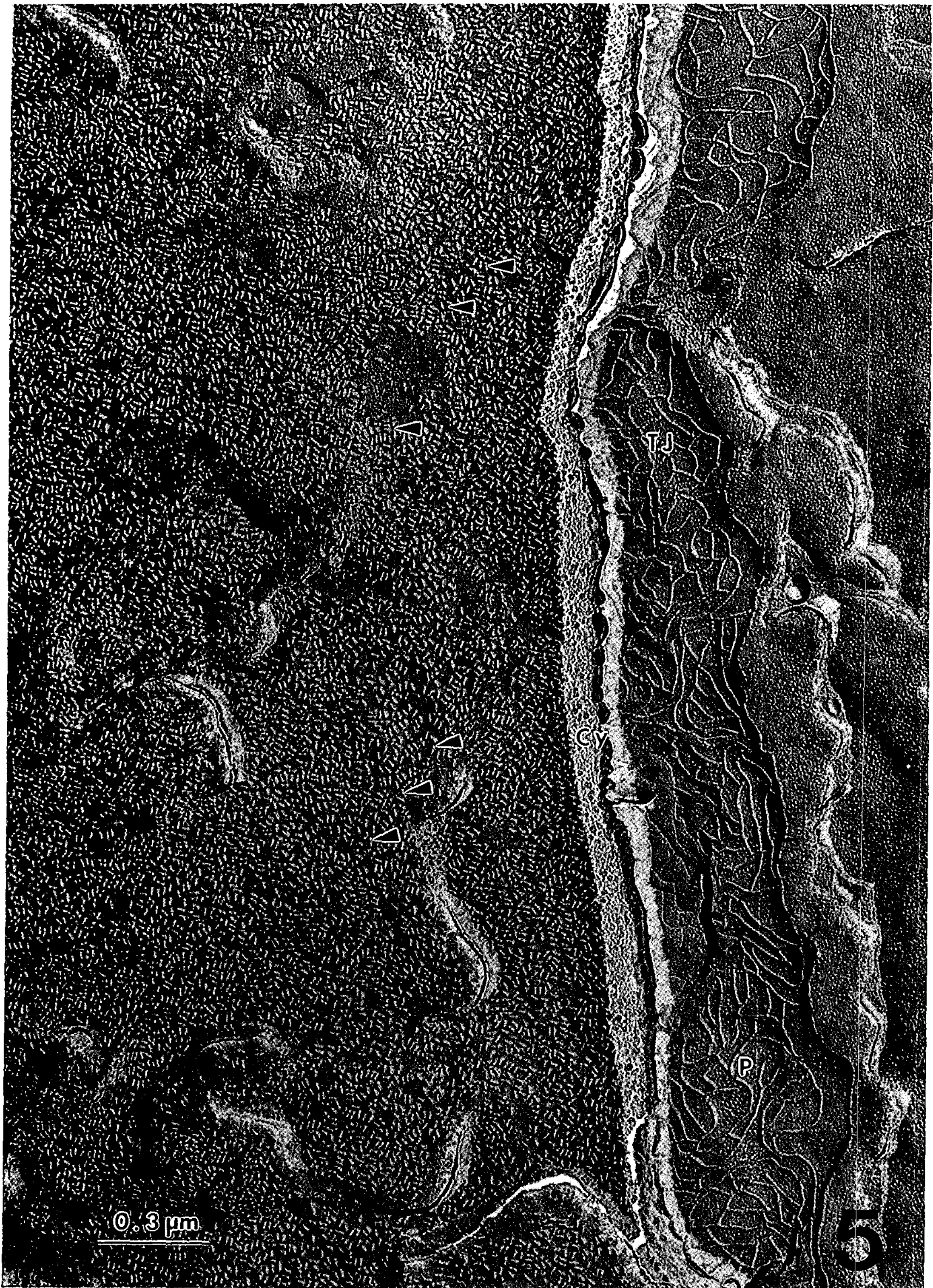



1 μm


TJ
Cy
P
0.3 μm
5