

# Inhibition of Amiloride-sensitive Apical $\text{Na}^+$ Conductance by Acetylcholine in Rabbit Cortical Collecting Duct Perfused In Vitro

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## Abstract

We examined effects of acetylcholine (ACh) on the electrical parameters and intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) in the isolated rabbit cortical collecting duct (CCD) perfused in vitro using the conventional microelectrode technique and microscopic fluorescence spectrophotometry. ACh ( $10^{-8}$  to  $10^{-5}$  M) in the bath caused a positive deflection of the transepithelial voltage ( $V_T$ ) and an increase in  $[\text{Ca}^{2+}]_i$ . Carbachol also showed similar but smaller effects. The effects of ACh were antagonized by muscarinic receptor antagonists. ACh at  $10^{-6}$  M hyperpolarized the apical membrane voltage and increased the fractional resistance of the apical membrane of the collecting duct cells accompanied by a positive deflection of  $V_T$  and an increase in transepithelial resistance, whereas it did not affect these parameters in the  $\beta$ -intercalated cells. In the presence of  $10^{-5}$  M amiloride in the lumen, the effects of ACh were almost completely abolished. The ACh-induced increase in  $[\text{Ca}^{2+}]_i$  is accounted for by the release of  $\text{Ca}^{2+}$  from intracellular store and  $\text{Ca}^{2+}$  entry from the bath. In the absence of  $\text{Ca}^{2+}$  in the bath, the ACh-induced changes in electrophysiological parameters were significantly smaller than those observed in the presence of  $\text{Ca}^{2+}$ . Both phorbol-12-myristate-13-acetate (PMA) and phorbol-12,13-dibutylate (PDBu), activators of protein kinase C (PKC), also inhibited the apical  $\text{Na}^+$  conductance. In the presence of PMA or PDBu in the bath, ACh did not show further inhibitory effect. 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine, an inhibitor of PKC, partially attenuated the effect of ACh. These observations indicate that ACh inhibits the apical  $\text{Na}^+$  conductance partly by both increasing  $[\text{Ca}^{2+}]_i$  and activating PKC. Such an action of ACh may partially explain its natriuretic effect. (*J. Clin. Invest.* 1994. 93:2649-2657.)  
Key words: acetylcholine •  $\text{Na}^+$  transport • cortical collecting duct • intracellular  $\text{Ca}^{2+}$  • protein kinase C

## Introduction

Infusion of acetylcholine (ACh)<sup>1</sup> into the renal artery has been demonstrated to increase urinary excretion of water and  $\text{Na}^+$ .

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1. Abbreviations used in this paper: ACh, acetyl choline; CCh, carbamyl choline; CCD, cortical collecting duct; CD cell, collecting duct cell;  $fR_A$ , fractional apical membrane resistance; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; IC cell, intercalated cell; PDBu,

Although such maneuver consistently increased renal plasma flow and decreased renal arteriolar resistance, it caused variable or no changes in GFR (1, 2). Therefore, this phenomenon has been thought to be ascribed not only to changes in renal hemodynamics but also to an inhibition of the tubular  $\text{Na}^+$  reabsorption.

Recently, several lines of evidence have accumulated in support of the view that the kidney is innervated by parasympathetic nerves and that ACh receptors exist in the nephrons. Pirola and associates (3, 4) reported that the outer and inner cortical cells of dog kidney exhibited the specific binding to [<sup>3</sup>H]quinuclidinyl benzilate (QNB). An autoradiographic study also showed that muscarinic receptors were localized in the cortical and medullary tubules of the rat kidney (5). McArdle et al. (6) showed that carbachol (CCh) stimulated phosphoinositide hydrolysis in the rabbit medullary tubules. Although these early studies did not show exact nephron segments having ACh receptors, McArdle et al. (7) demonstrated the specific [<sup>3</sup>H]QNB binding in the rabbit inner medullary collecting duct (CD) cells. Marchetti et al. (8) reported that both CCh and ACh increased intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) in the rat outer medullary collecting tubules. These findings indicate that muscarinic receptors exist, if not exclusively, in the CD.

Functional significance of muscarinic receptors in the CD has not been fully understood. CCh has been demonstrated to inhibit the arginine vasopressin-stimulated water flow in the rabbit cortical collecting duct (CCD) by increasing  $[\text{Ca}^{2+}]_i$  (9). In the toad bladder, both ACh and CCh have been shown to inhibit  $\text{Na}^+$  transport (10). It has been also reported that CCh inhibits  $\text{NaCl}$  absorption in ileal villus cells (11). Therefore, it is possible that ACh may also inhibit  $\text{Na}^+$  transport in the renal tubule. However, it has never been tested whether muscarinic agonists inhibit  $\text{Na}^+$  transport in the collecting duct. The  $\text{Na}^+$  transport in the CCD is thought to occur through both the  $\text{Na}^+$  channel in the apical membrane and the  $\text{Na}^+$ - $\text{K}^+$  ATPase in the basolateral membrane. The aim of the present study was to clarify whether ACh directly affected the  $\text{Na}^+$  transport in the CCD. Therefore, we investigated the effect of ACh on the function of the rabbit CCD perfused in vitro using the conventional microelectrode technique and the microscopic fluorescence spectrophotometry method.

## Methods

*In vitro microperfusion of isolated tubules.* The segments of isolated CCD were perfused in vitro according to the method of Burg et al. (12) with slight modifications as reported previously from our laboratory (13, 14). In brief, segments of the CCD were isolated from male Japa-

phorbol-12,13-dibutylate; PKC, protein kinase C; QNB, quinuclidinyl benzilate;  $R_T$ , transepithelial resistance;  $V_B$ , basolateral membrane voltage;  $V_T$ , transepithelial voltage.

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nese white rabbits weighing 1.5–3.0 kg. Animals were maintained on a regular rabbit laboratory diet and allowed free access to tap water. Rabbits were anesthetized with pentobarbital (35 mg/kg, i.v.) and their kidneys were removed. Then thin kidney slices were made and transferred to a cooled dish (4–6°C) containing modified Collins' solution containing (in mM); 14 KH<sub>2</sub>PO<sub>4</sub>, 44 K<sub>2</sub>HPO<sub>4</sub>, 14 KCl, 8 NaHCO<sub>3</sub>, and 160 sucrose (pH 7.4). The CCD segment was dissected with fine forceps under a stereomicroscope.

An isolated tubule was transferred to a bath fixed on an inverted microscope (IMT-2, Olympus, Tokyo) equipped with a set of microperfusion system (Narishige, Tokyo) and perfused in vitro as previously described (13, 14). The tubular lumen was perfused at a rate of 10–20 nl/min by hydrostatic pressure by varying the height of the fluid reservoir which was connected to the back end of the perfusion pipette. To achieve rapid exchange of the bathing solution, the perfusion chamber was made to have a volume of 0.1 ml having a runoff groove in series. The temperature of the bathing solution was kept at 37°C by adjusting the temperature of the heating jacket. The flow rate of the bathing solution was adjusted at ~8–10 ml/min, allowing the bathing solution to be exchanged within 10 s.

**Electrical measurements.** The transepithelial voltage ( $V_T$ ) and the voltage deflection at the proximal end of the tubule ( $\Delta V_0$ ) were measured through a microelectrode transmurally punctured into the tubular lumen, which was connected to one channel of a dual electrometer (Duo 773, WP Instruments, New Haven, CT). A flowing boundary 3 M KCl-agar bridge which was connected to a calomel half cell was placed at the outflow of the bath and served as a system ground. Through the perfusion pipettes current ( $I_0$ ) was injected into the tubular lumen when the transepithelial resistance ( $R_T$ ) and the fractional resistance of the apical membrane ( $fR_A$ ) were measured. The advantage of separating current-injection and voltage-measuring channels in the perfusion pipettes has been described by other investigators (15, 16). The current (usually 50–100 nA) was injected by a stimulator (SEN-7103, Nihon Kodens, Tokyo) with the other channel of electrometer (Duo773) which has a built-in stimulus isolator serving as a constant current source. Voltage deflection at the distal end of the tubule ( $\Delta V_L$ ) was measured by a 3 M KCl-agar bridge inserted into the collecting pipette, which was connected to a hand made high impedance voltage follower.

The transepithelial resistance was measured by finite cable analysis assuming that both terminals of the cable were closed (15, 16). The following six cable equations were used. The tubular length constant ( $\lambda$ ) was calculated from

$$\lambda = L / \cosh^{-1} (\Delta V_0 / \Delta V_L), \quad (1)$$

where  $\Delta V_0$  and  $\Delta V_L$  are the voltage deflections of the proximal and the distal end of the perfused tubule, respectively.  $L$  is the cable length of the perfused tubule. The specific transepithelial resistance was measured as

$$R_T = 2\pi\lambda(\Delta V_0 / I_0) \tanh(L/\lambda), \quad (2)$$

where  $I_0$  is a current pulse injected into tubular lumen. Because the voltage deflection at the proximal end of the tubule ( $\Delta V_0$ ) was a linear function of the injected current over the wide range from –200 to +200 nA, we have used the current of 50–100 nA with a duration of 500 ms. The core resistance ( $R_C$ ) and electrical radius ( $r_E$ ) were evaluated as

$$R_C = (\Delta V_0 / \lambda I_0) \tanh(L/\lambda) \quad (3)$$

$$r_E = (\rho / \pi R_C)^{1/2}, \quad (4)$$

where  $\rho$  is volume resistivity of the luminal fluid. The electrical radius was compared to the optical radius ( $\rho_O$ ) as a check of the internal consistency of the cable analysis technique. Experiments with ratio of  $\rho_E / \rho_O$  between 0.8 and 1.2 were accepted as being reliable.

The basolateral membrane potential ( $V_B$ ) was measured with conventional microelectrodes fabricated as previously (13, 14). They were filled with 0.5 M KCl solution and had resistance of 120–180 M $\Omega$  and

negligible tip potential (<5 mV). They were fixed to a microelectrode holder containing Ag/AgCl-pellet and connected to a high-impedance electrometer (model 8100, single-electrode system, Dagan, Minneapolis, MN). To impale tubular cells, a microelectrode was positioned against the basolateral membrane with a hydraulic micromanipulator (MO-102N-1, Narishige), which was mounted on another micromanipulator (MN-1, Narishige) fixed to the stage of the inverted microscope. The microelectrode was advanced into the cell by using current oscillations (“Buzz”).

Fractional resistance of the apical membrane ( $fR_A$ ) was calculated from

$$fR_A = 1 - \Delta V_B / \Delta V_X, \quad (5)$$

where  $\Delta V_B$  is the current-induced voltage deflection of the basolateral membrane potential. The deflection of transepithelial voltage at the site of microelectrode puncture ( $\Delta V_X$ ) was calculated as

$$\Delta V_X = \Delta V_0 \cosh((L - X)/\lambda) / \cosh(L/\lambda), \quad (6)$$

where  $X$  is the axial distance between the mouth of the current injecting pipette and the puncture site with a microelectrode. All voltages ( $V_T$ ,  $V_L$ , and  $V_B$ ) were recorded on a multipen recorder (R-306, Rikadenki, Tokyo). Figures depicted in the present manuscript were digitized from original trace records through an image scanner (PC-IN506, NEC, Tokyo), and redrawn with a laser printer (Laser Jet III, Yokogawa-Hewlett-Packard, Tokyo).

**Peanut agglutinin (PNA) binding study.** Fluorescence of perfused tubules was observed by an inverted fluorescence microscope (OSP-3, Olympus). To identify  $\beta$ -intercalated ( $\beta$ -IC) cells, FITC-labeled PNA (10  $\mu$ g/ml) was loaded from the lumen for 5 min at 37°C (17). After loading, the tubules were excited at 490 nm and observed under high magnification. A cell with a characteristic lectin cap on the edge of the tubule was identified as a  $\beta$ -IC cell. To test the effect of ACh on  $\beta$ -IC cell, we impaled the microelectrode into the PNA-positive cell.

**Intracellular Ca<sup>2+</sup> concentration measurements.** We measured [Ca<sup>2+</sup>]<sub>i</sub> using fura 2, a fluorescent Ca<sup>2+</sup> indicator, as previously reported (14, 18, 19). Isolated microperfused tubules were incubated in 200  $\mu$ l of standard bicarbonate buffer solution containing 20  $\mu$ M acetoxyethyl ester of fura 2 (fura 2-AM, Dojin Biochemical, Kumamoto) at room temperature (22–25°C) for 20 min. Intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) was measured using a fluorescence microscope photometry system (OSP-3). Tubular cells were excited at 340 and 380 nm and emission was monitored at 510 nm. The fluorescent dye was excited alternately at 340 and 380 nm by spinning the sector mirror at a rate of 333 rpm. In this apparatus, it takes at least 10 ms to obtain one fluorescence ratio ( $R = F_{340} / F_{380}$ ). One data point represented an arithmetical average of 100 fluorescence ratios for sampling time of 1 s. All the procedure were controlled by a computer (HP9000, model 332, Hewlett-Packard, Fort Collins, CO) and the data were plotted on a laser printer (HP Laser Jet III). [Ca<sup>2+</sup>]<sub>i</sub> was calculated based on the formula described by Grynkiewicz et al. (20) as specified below:

$$[\text{Ca}^{2+}]_i = \frac{(R - R_{\min})F_{\max}K_d}{(R_{\max} - R)F_{\min}} \quad (7)$$

where  $R_{\min}$  is the ratio at zero calcium in the bathing fluid and  $R_{\max}$  is the ratio at saturated calcium concentration.  $F_{\max}$  is the ratio at saturated calcium concentration. The effective dissociation constant  $K_d$  of 224 nM for the fura 2–Ca<sup>2+</sup> complex was used as reported by Grynkiewicz et al. (20). Because it has been reported that  $K_d$  of fura 2 for Ca<sup>2+</sup> varies in the presence of protein, absolute Ca<sup>2+</sup> concentrations thus calculated might not be accurate enough. We report the concentration values to provide information on relative changes in Ca<sup>2+</sup> concentration. Leakage of fura 2 from cells was <5% for 15 min and the autofluorescence on tubule was <10% compared to fluorescence of fura 2-AM-loaded tubules.

**Solutions and drugs.** The perfusate and the bathing solution were modified bicarbonate Krebs-Ringer solution (standard solution) containing (in mM): 115 NaCl, 5 KCl, 25 NaHCO<sub>3</sub>, 0.8 Na<sub>2</sub>HPO<sub>4</sub>, 0.2

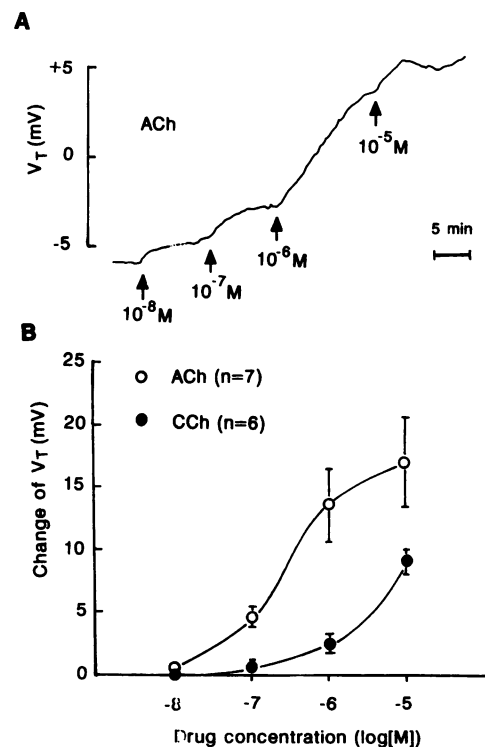
NaH<sub>2</sub>PO<sub>4</sub>, 1.8 CaCl<sub>2</sub>, 1.0 MgCl<sub>2</sub>, 10 Na acetate, 8.3 D-glucose, 5.0 L-alanine. The Ca<sup>2+</sup>-free solution was made by omitting CaCl<sub>2</sub> from and adding 1.0 mM EGTA to the standard solution. The 12 mM Cl<sup>-</sup> solution was made by replacement of 110.8 mM NaCl with equimolar Na cyclamate. All the solution were continuously bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub> to maintain the pH at 7.4. All the chemicals were purchased from respective companies listed as follows: acetylcholine from Wako Jyunyaku (Tokyo, Japan); atropine, pirenzepine, carbachol, amiloride, PMA, and phorbol-12,13-dibutylate (PDBu) from Sigma Chemical Co (St. Louis, MO); and H-7 from Seikagaku Kogyo (Tokyo). In the experiments using PMA and PDBu which were dissolved in DMSO, the final concentration of DMSO in the bathing solution was made constant (0.1%) throughout the experiments.

**Statistical analysis.** All the data were expressed as means±SE. Statistical analyses were performed by either paired or nonpaired *t* test, when appropriate. The *P* value <0.05 was considered to be significant.

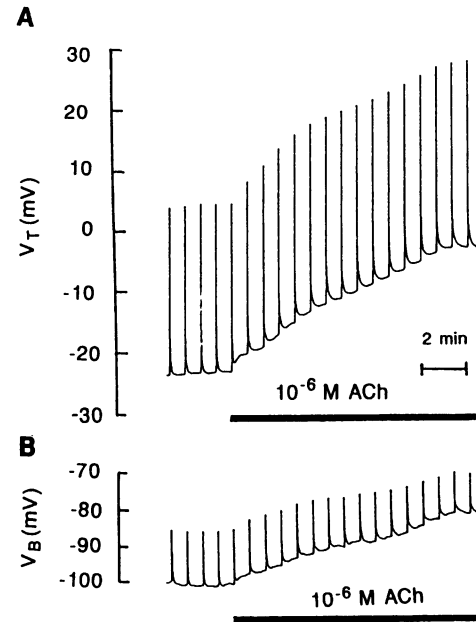
## Results

When the isolated CCDs were perfused with artificial physiological solution at the rate of 10–20 nl/min, they showed electrophysiological characteristics similar to those reported previously with respect to  $V_T$ ,  $V_B$ ,  $fR_A$ , and  $R_T$  (21, 22). The averaged inner diameter was  $25.0 \pm 0.6 \mu\text{m}$  ( $n = 52$ ). The mean values of the tubular length and the length constant were  $936.1 \pm 30.1 \mu\text{m}$  ( $n = 52$ ) and  $271.6 \pm 8.6 \mu\text{m}$  ( $n = 52$ ), respectively.

**Effect of ACh on electrical parameters in CD cell.** Addition of ACh in the bath decreased lumen-negative  $V_T$  in the in vitro



**Figure 1.** Effects of ACh and CCh on  $V_T$  in rabbit CCD. A representative tracing of the ACh-induced change in  $V_T$  is shown in A. ACh was cumulatively applied to the bathing solution. Dose–response relationships of the effects of ACh and CCh on  $V_T$  are shown in B. The amplitude of the agonists-induced changes of  $V_T$  are plotted against each concentration of agonists. Each points are expressed as mean±SE. Numbers of observations are shown in B.



**Figure 2.** Representative tracings of the ACh-induced changes in electrophysiological parameters in the rabbit CCD.  $V_T$  and  $V_B$  are shown in A and B, respectively. ACh was applied in the bath during the period indicated by solid bar in each panel.

perfused rabbit CCD in a concentration-dependent manner ( $10^{-8}$  to  $10^{-5}$  M, Fig. 1). To clarify the ionic mechanism of the ACh-induced positive deflection of  $V_T$ , we performed the cable analysis. Application of  $10^{-6}$  M ACh to the bathing solution resulted in marked positive deflection of  $V_T$  and depolarization of  $V_B$  in the CD cell (Fig. 2, Table I).  $V_A$  significantly hyperpolarized from  $-70.5 \pm 4.1$  to  $-72.7 \pm 3.4$  mV ( $n = 8$ ,  $P < 0.05$ , paired *t* test). Furthermore, both  $R_T$  and  $fR_A$  were increased by ACh (Fig. 2, Table I). These observations indicate that ACh decreases the ionic conductance of the apical membrane of the CD cell.

**Effect of ACh on electrical parameters in the  $\beta$ -IC cell.** We also examined the effect of ACh on the electrophysiological parameters in  $\beta$ -IC cell which was another major cell population in the rabbit CCD (22, 23). In  $\beta$ -IC cells,  $10^{-6}$  M ACh applied in the bath did not change  $V_B$  and  $fR_A$ , indicating that  $\beta$ -IC cells do not contribute to the ACh-induced positive deflection of  $V_T$ .

**Table I.** Effect of ACh on Electrophysiological Parameters in Rabbit CCD

	Control	ACh	<i>n</i>	<i>P</i>
$V_T$ (mV)	$-16.9 \pm 2.5$	$-6.7 \pm 1.6$	19	<0.001
$R_T$ ( $\Omega\text{cm}^2$ )	$94.9 \pm 7.1$	$111.2 \pm 9.7$	19	<0.001
CD cell				
$V_B$ (mV)	$-87.6 \pm 4.5$	$-79.0 \pm 3.4$	8	<0.01
$fR_A$	$0.36 \pm 0.06$	$0.51 \pm 0.06$	8	<0.01
$\beta$ -IC cell				
$V_B$ (mV)	$-15.9 \pm 2.3$	$-13.4 \pm 1.6$	3	NS
$fR_A$	$0.94 \pm 0.01$	$0.95 \pm 0.01$	3	NS

Values are expressed as means±SE.

In the connecting tubule (CNT), there are two distinct types of cells: CNT and  $\beta$ -IC (22, 24). To ascertain that the  $\beta$ -IC cell does not respond to ACh, the effects of ACh on  $V_T$  and  $R_T$  were examined in the CNT. Bath application of  $10^{-6}$  M ACh did not affect  $V_T$  ( $-10.0 \pm 1.3$  to  $-9.8 \pm 1.3$  mV;  $n = 3$ , NS) and  $R_T$  ( $16.1 \pm 6.5$  to  $16.6 \pm 6.1$   $\Omega\text{cm}^2$ ;  $n = 3$ , NS) in the CNT, indicating that the  $\beta$ -IC cell as well as the CNT cell does not participate in the ACh-induced positive deflection of  $V_T$ .

**Effect of amiloride on the action of ACh.** The inhibition of the apical  $\text{Na}^+$  channel seemed to be the most plausible mechanism to account for all the ACh-induced changes of electrophysiological parameters. To confirm whether the ACh-induced responses were ascribed to the inhibition of the apical  $\text{Na}^+$  channel, we investigated the effect of amiloride, a  $\text{Na}^+$  channel blocker, on the action of ACh. Application of  $10^{-5}$  M amiloride in the perfusate caused a positive deflection of  $V_T$ , a depolarization of  $V_B$ , and a hyperpolarization of  $V_A$  by  $7.6 \pm 3.9$  mV ( $P < 0.05$ ,  $n = 4$ ). Both  $R_T$  and  $fR_A$  were increased by amiloride (Table II). These results confirmed that amiloride blocked the apical  $\text{Na}^+$  conductance. In the presence of  $10^{-5}$  M amiloride in the lumen, ACh did not change  $V_T$ ,  $V_B$ , and  $fR_A$  (Table II), indicating that ACh-induced changes in electrical parameters are due to the inhibition of apical  $\text{Na}^+$  conductance in the CCD.

**Effect of ACh on intracellular calcium concentration.** Bath application of ACh evoked a transient increase in  $[\text{Ca}^{2+}]_i$  followed by a sustained one in a concentration-dependent fashion ( $10^{-8}$  to  $10^{-5}$  M, Fig. 3). Concentration dependency of the action on  $[\text{Ca}^{2+}]_i$  was very similar to that on  $V_T$  (Fig. 1), suggesting that the ACh-induced response is mediated by cytosolic free  $\text{Ca}^{2+}$ .

To determine the source of increased  $\text{Ca}^{2+}$ , we examined the effects of  $\text{Ca}^{2+}$  removal from either lumen or bath on the ACh-induced increase in  $[\text{Ca}^{2+}]_i$ . We measured the magnitude of the ACh-induced increases in  $[\text{Ca}^{2+}]_i$  at the peak and 2 min after the initiation of the response that were considered to reflect a transient and a sustained component of the ACh-induced increases in  $[\text{Ca}^{2+}]_i$ , respectively.  $\text{Ca}^{2+}$  removal from the lumen did not alter  $[\text{Ca}^{2+}]_i$ . In the absence of  $\text{Ca}^{2+}$  in the luminal solution, ACh evoked a rise in  $[\text{Ca}^{2+}]_i$  (peak  $102.3 \pm 11.9$  nM, after 2 min  $76.9 \pm 10.2$  nM;  $n = 5$ ) to a similar extent observed in the presence of  $\text{Ca}^{2+}$  (peak  $111.1 \pm 11.8$  nM, after 2 min  $76.2 \pm 9.9$  nM;  $n = 5$ , Fig. 4). In contrast, only a small and transient increase in  $[\text{Ca}^{2+}]_i$  was evoked by ACh in the absence of  $\text{Ca}^{2+}$  in the bathing solution (peak  $55.0 \pm 13.0$  nM, after 2 min  $-5.6 \pm 1.6$  nM;  $n = 6$ ), whereas, after repletion of  $\text{Ca}^{2+}$  to the bath, ACh evoked greater responses in a tran-

Table II. Effect of ACh on Electrophysiological Parameters in the Presence of Amiloride in the Lumen in Rabbit CCD

	Control	Amiloride	Amiloride + ACh
$V_T$ (mV)	$-17.1 \pm 3.0$ (6)	$3.3 \pm 0.8$ (6) <sup>‡</sup>	$3.6 \pm 1.0$ (6)
$V_B$ (mV)	$-88.4 \pm 5.1$ (4)	$-73.6 \pm 3.3$ (4)*	$-70.5 \pm 5.1$ (4)
$R_T$ ( $\Omega\text{cm}^2$ )	$77.3 \pm 3.3$ (6)	$82.9 \pm 2.7$ (6)*	$91.6 \pm 5.1$ (6)*
$fR_A$	$0.26 \pm 0.06$ (4)	$0.44 \pm 0.08$ (4)*	$0.57 \pm 0.13$ (4)

Concentrations of amiloride and ACh were  $10^{-5}$  and  $10^{-6}$  M respectively. Values are expressed as means  $\pm$  SE. They were compared with the control using the paired *t*-test. \*  $P < 0.05$ ; <sup>‡</sup>  $P < 0.001$ .

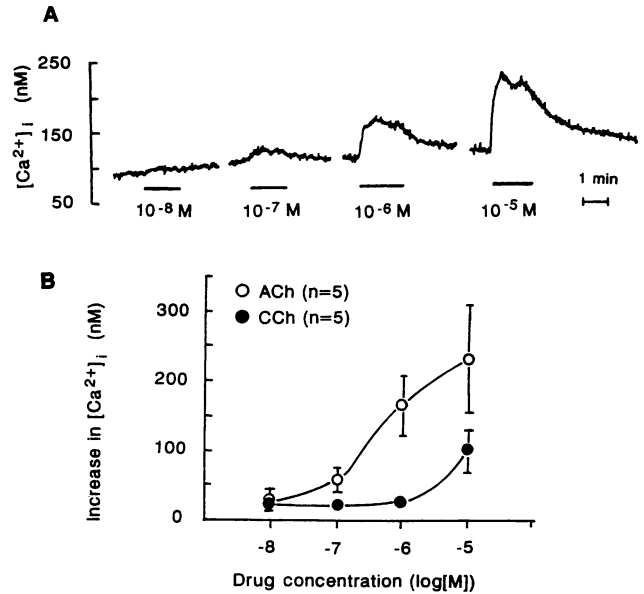


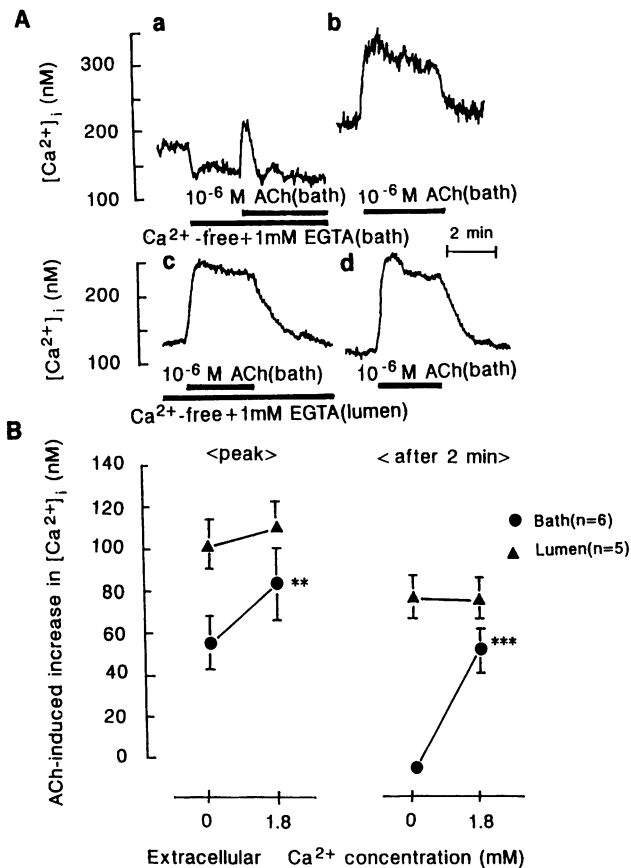
Figure 3. Effects of ACh and CCh on  $[\text{Ca}^{2+}]_i$  in the rabbit CCD. Representative tracings of the ACh-induced increase in  $[\text{Ca}^{2+}]_i$  are shown in A. In B, dose-response relationships of ACh and CCh for  $[\text{Ca}^{2+}]_i$  are shown. Each point represents mean  $\pm$  SE. Numbers of observations are shown in B.

sient increase in  $[\text{Ca}^{2+}]_i$  as well as in a sustained one (peak  $83.0 \pm 17.2$  nM, after 2 min  $51.1 \pm 10.8$  nM;  $n = 6$ , Fig. 4). These observations suggest that the ACh-induced increase in  $[\text{Ca}^{2+}]_i$  occurs through two distinct pathways: a release from intracellular  $\text{Ca}^{2+}$  store site and an influx through the basolateral membrane.

**Characterization of the target of ACh action.** In the next series of experiments, we examined effects of muscarinic antagonists, atropine and pirenzepine, on the ACh-induced responses. In the absence of antagonists, the positive deflection of  $V_T$  to  $10^{-6}$  M ACh was very reproducible when ACh was applied twice. The magnitude of the ACh-induced  $V_T$  changes for the first and second challenges were  $7.9 \pm 0.6$  and  $7.0 \pm 0.9$  mV (Fig. 5,  $n = 7$ , NS), respectively. Treatments by muscarinic antagonists before the second application of ACh significantly attenuated the ACh-induced responses. Atropine or pirenzepine both at  $10^{-6}$  M decreased the ACh-induced changes of  $V_T$  from  $7.8 \pm 2.1$  to  $1.3 \pm 0.7$  mV ( $n = 4$ ,  $P < 0.05$ ) or from  $11.6 \pm 3.2$  to  $2.8 \pm 1.3$  mV ( $n = 4$ ,  $P < 0.05$ ), respectively (Fig. 5).

Both muscarinic antagonists also exhibited similar inhibitory effects on the ACh-induced increase in  $[\text{Ca}^{2+}]_i$ . They did not alter the baseline  $[\text{Ca}^{2+}]_i$ . In the presence of  $10^{-6}$  M atropine in the bath, ACh evoked only a small rise in  $[\text{Ca}^{2+}]_i$  (peak  $5.2 \pm 1.2$  nM;  $n = 5$ ), whereas it clearly caused a greater increase in  $[\text{Ca}^{2+}]_i$  after removal of atropine (peak  $66.4 \pm 18.7$  nM;  $n = 5$ ,  $P < 0.05$  vs. the value in the presence of atropine, Fig. 6). Although in the presence of  $10^{-6}$  M pirenzepine in the bath, ACh produced a significant increase in  $[\text{Ca}^{2+}]_i$  (peak  $26.6 \pm 9.1$  nM;  $n = 5$ ), in the absence of pirenzepine ACh caused much greater increase in  $[\text{Ca}^{2+}]_i$  (peak  $91.9 \pm 30.8$  nM;  $n = 5$ ,  $P < 0.05$  vs. the value in the presence of pirenzepine, Fig. 6).

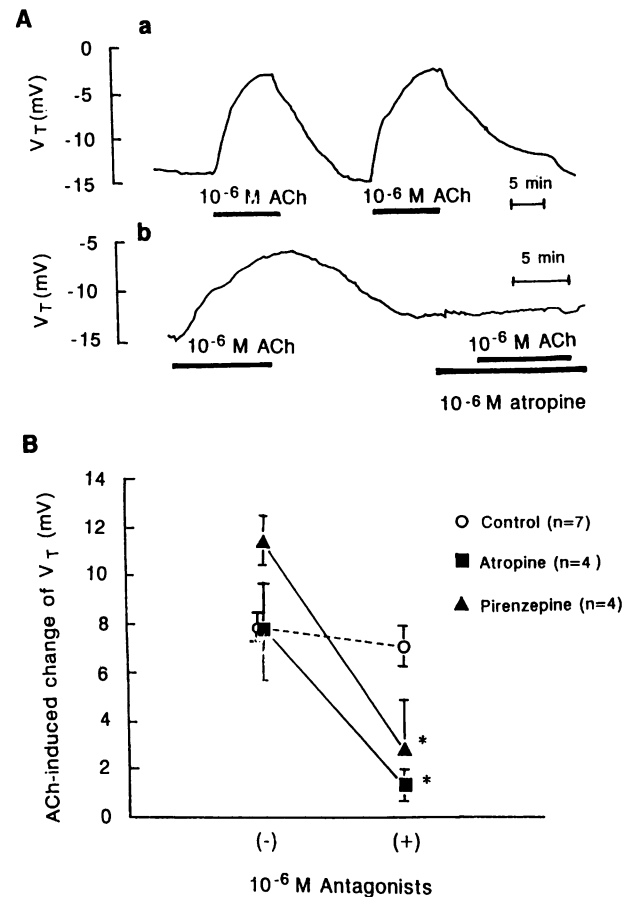
**Roles of calcium in the action of ACh.** To ascertain whether the ACh-induced changes in electrical parameters depended on the  $\text{Ca}^{2+}$  in the peritubular fluid, we investigated the effect of



**Figure 4.** Effect of Ca<sup>2+</sup>-removal from bath or lumen on the ACh-induced increase in [Ca<sup>2+</sup>]<sub>i</sub>. Representative tracings are shown in *A*. Bath application of 10<sup>-6</sup> M ACh was repeated in the same tubule for two times. First and second responses were observed in the absence and presence of Ca<sup>2+</sup>, respectively. Ca<sup>2+</sup> was removed either from the bath or from the lumen. Periods of removal of Ca<sup>2+</sup> and application of ACh are shown by solid bars in *A*. Note that Ca<sup>2+</sup> removal from the bath decreased basal [Ca<sup>2+</sup>]<sub>i</sub> and inhibited the sustained increase in [Ca<sup>2+</sup>]<sub>i</sub> in the presence of ACh. Summarized data are shown in *B*. The amplitude of the ACh-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> at peak and 2 min after the initiation of the response were measured. Each point represents mean±SE. Numbers of observations are indicated in panel *B*. \*\**P* < 0.01; \*\*\**P* < 0.005.

ACh on V<sub>T</sub> and R<sub>T</sub> in the absence of Ca<sup>2+</sup> in the bath (0 mM Ca<sup>2+</sup> + 1 mM EGTA). Because we could not maintain the stable impalement of microelectrode in the CD cell in the Ca<sup>2+</sup>-free condition, we monitored only V<sub>T</sub> and R<sub>T</sub>. Removal of Ca<sup>2+</sup> from the bathing solution did not alter both V<sub>T</sub> (from -11.8±1.0 to -11.5±1.0 mV; *n* = 7, NS) and R<sub>T</sub> (from 84.8±6.9 to 83.1±6.5 Ωcm<sup>2</sup>; *n* = 7, NS). As shown in Fig. 7, only a small change of V<sub>T</sub> (from -11.5±1.0 to -8.7±1.4 mV; *n* = 7, *P* < 0.05) and a tendency to increase R<sub>T</sub> (from 83.1±6.5 to 86.2±8.9 Ωcm<sup>2</sup>; *n* = 7, NS) were observed by 10<sup>-6</sup> M ACh in the absence of Ca<sup>2+</sup> in the bath. Compared with the control group shown in Table I, removal of Ca<sup>2+</sup> from the bathing solution significantly reduced the ACh-induced responses (Fig. 7), indicating that ACh requires Ca<sup>2+</sup> influx through the basolateral membrane to fully evoke the changes in electrophysiological parameters in the CCD.

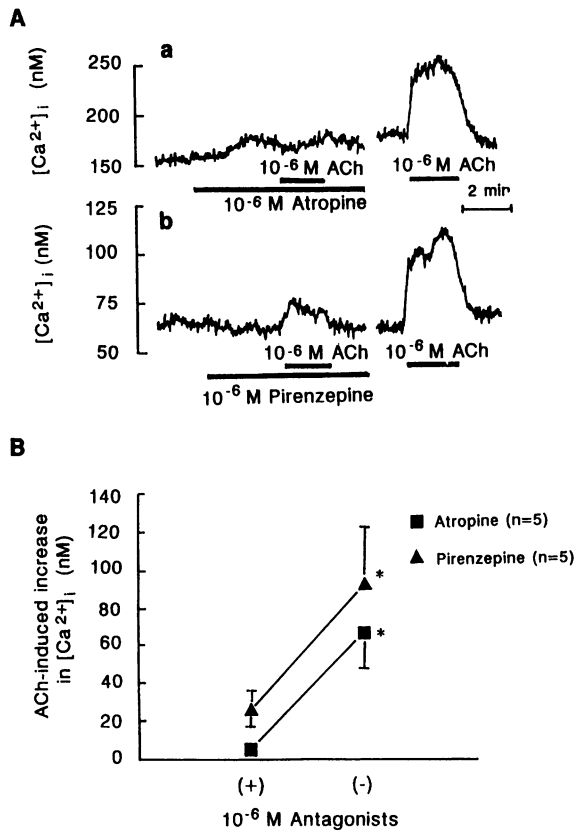
*Effects of modulators of protein kinase C (PKC) on the action of ACh.* It has been shown that phorbol esters, activators of PKC, applied in the bath reduced lumen-negative V<sub>T</sub> and



**Figure 5.** Effect of muscarinic receptor antagonists on the ACh-induced negative deflection of V<sub>T</sub> in the rabbit CCD. Representative tracings are shown in *A*. Bath application of 10<sup>-6</sup> M ACh was repeated twice in the same tubule. In second challenge, ACh was applied in the absence (-) or presence (+) of 10<sup>-6</sup> M atropine in the bath. Drugs were applied during the period indicated by solid bars. Summarized data are shown in *B*. Each point represents mean±SE. Numbers of observations are shown in *B*. \**P* < 0.05.

inhibited Na<sup>+</sup> reabsorption in the rabbit CCD (25). It has been also reported that PGE<sub>2</sub> inhibits the apical Na<sup>+</sup> channel via the PKC pathway (26). Therefore, we tested the effects of phorbol esters, PMA and PDBu (Fig. 8), and H-7, an inhibitor of PKC (Fig. 9), on the ACh-induced responses.

Either PMA or PDBu added in the bath at the concentration of 10<sup>-7</sup> M shifted V<sub>T</sub> from -11.5±2.2 to -4.0±1.0 mV (*n* = 7, *P* < 0.01) and from -14.3±1.3 to -6.0±1.2 mV (*n* = 4, *P* < 0.005) and increased R<sub>T</sub> from 79.1±5.4 to 85.5±4.7 Ωcm<sup>2</sup> (*n* = 7, *P* < 0.05) and from 74.4±6.0 to 84.1±6.8 Ωcm<sup>2</sup> (*n* = 4, *P* < 0.05), respectively (Fig. 8). These actions of phorbol esters were completely attenuated by luminal application of 10<sup>-5</sup> M amiloride. In the presence of amiloride in the lumen, PMA did not change V<sub>T</sub> (from 2.2±0.9 to 1.4±0.6 mV; *n* = 5, NS) or R<sub>T</sub> (from 86.9±7.2 to 91.9±8.9 Ωcm<sup>2</sup>; *n* = 5, NS). Under the same condition, PDBu did not alter V<sub>T</sub> (from 1.0±0.4 to 0.9±0.5 mV, *n* = 4, NS) or R<sub>T</sub> (from 73.8±11.2 to 75.3±11.2 Ωcm<sup>2</sup>; *n* = 4, NS) as well. These observations confirmed that the responses induced by PMA or PDBu were mainly due to inhibition of the apical Na<sup>+</sup> channel. After the treatment with 10<sup>-7</sup> M PMA for 10–15 min, 10<sup>-6</sup> M ACh produced only a slight change in V<sub>T</sub> (from -4.0±1.0 to -3.0±1.0 mV; *n* = 7, *P*



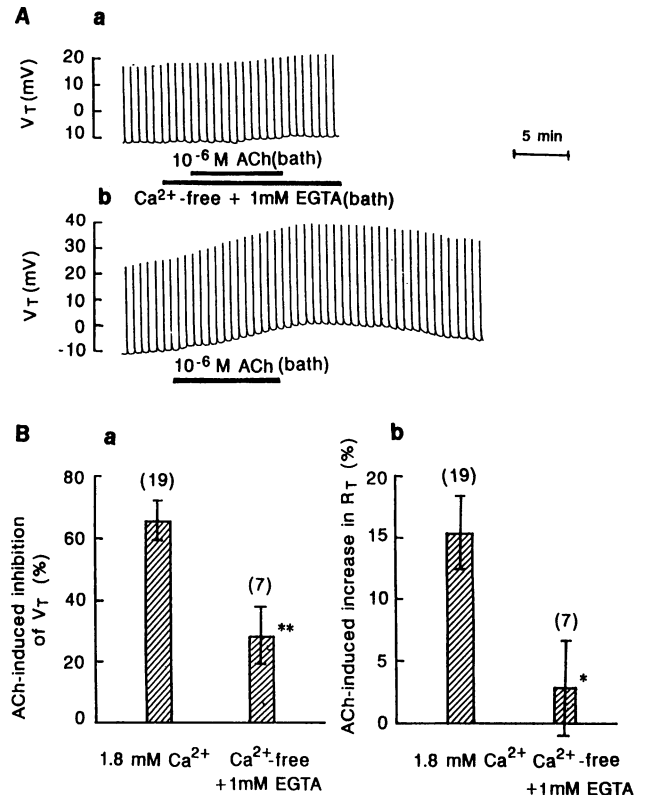
**Figure 6.** Effects of muscarinic receptor antagonists on the ACh-induced increase in  $[Ca^{2+}]_i$ . Typical tracings are shown in *A*. ACh at  $10^{-6}$  M was applied in the bath in the same tubule for two times. First and second responses were observed in the presence (+) and absence (-) of antagonist, respectively. Drugs were applied during the period indicated by solid bars. Summarized data are shown in *B*. Each point represents mean  $\pm$  SE. Numbers of observations are shown in *B*. \* $P < 0.05$ .

$< 0.05$ ) and no change in  $R_T$  (from  $85.5 \pm 4.7$  to  $87.1 \pm 5.3 \Omega\text{cm}^2$ ;  $n = 7$ , NS). Exposure to  $10^{-7}$  M PDBu from the bath also masked the ACh-induced responses ( $V_T$  from  $-6.0 \pm 1.2$  to  $-5.3 \pm 0.9$  mV,  $n = 4$ , NS;  $R_T$  from  $84.1 \pm 6.8$  to  $89.7 \pm 8.1 \Omega\text{cm}^2$ ,  $n = 4$ , NS).

Bath application of H-7 ( $3 \times 10^{-5}$  M) did not cause any changes of  $V_T$  (from  $-14.7 \pm 3.4$  to  $-15.5 \pm 3.0$  mV;  $n = 4$ , NS) and  $R_T$  (from  $87.5 \pm 16.6$  to  $86.2 \pm 15.4 \Omega\text{cm}^2$ ,  $n = 4$ , NS). In the presence of H-7 in the bath, addition of  $10^{-6}$  M ACh showed a tendency to decrease lumen-negative  $V_T$  (from  $-15.5 \pm 3.0$  to  $-10.5 \pm 2.6$  mV;  $n = 4$ , NS) and a significant increase in  $R_T$  (from  $86.2 \pm 15.4$  to  $92.0 \pm 16.0 \Omega\text{cm}^2$ ;  $n = 4$ ,  $P < 0.05$ ). Compared with the control group (shown in Table I), however, the action of  $10^{-6}$  M ACh was apparently reduced by H-7 (Fig. 9).

## Discussion

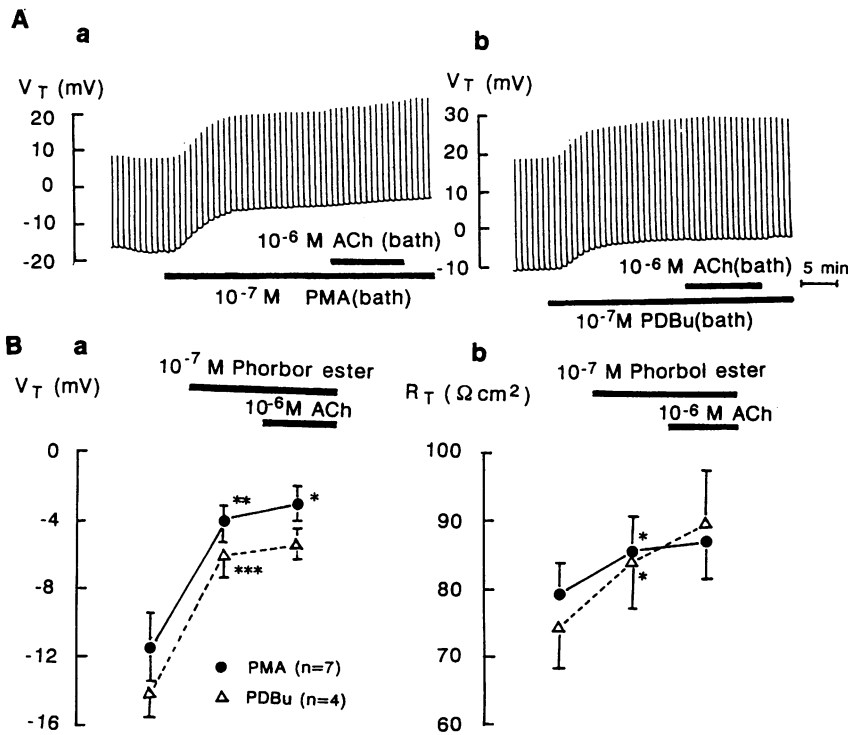
We reported here that ACh inhibited the amiloride-sensitive apical  $Na^+$  conductance in the CD cell of the rabbit CCD by an activation of muscarinic receptor located in the basolateral membrane and that the ACh-induced responses were mediated by the combination of an increase in  $[Ca^{2+}]_i$  and a PKC activation.



**Figure 7.** Effect of  $Ca^{2+}$ -removal from bath on the ACh-induced changes of  $V_T$  and  $R_T$ . Representative tracings are shown in *A*. Periods of removal of  $Ca^{2+}$  and application of ACh are shown by solid bars. The recordings shown in *Aa* and *Ab* were obtained from different tubules. Summarized data are shown in *B*. The ACh-induced inhibition of lumen-negative  $V_T$  (*Ba*) and the ACh-induced increase in  $R_T$  (*Bb*) are compared between the conditions in the presence and absence of  $Ca^{2+}$  in the bath. The data are expressed as mean  $\pm$  SE. Numbers of observations are indicated on the top of each column. \* $P < 0.05$ ; \*\* $P < 0.01$ , compared to the control values.

**Target cell of ACh in the rabbit CCD.** Three distinct types of cells (CD,  $\alpha$ -IC, and  $\beta$ -IC cells) can be identified in the rabbit CCD by the conventional microelectrode method (22). The CD cells possess a relatively high  $V_B$  and a low  $fR_A$ , whereas IC cells have a relatively low  $V_B$  and a high  $fR_A$  which is close to unity. Although IC-cells have high  $Cl^-$  conductance in the basolateral membrane,  $\alpha$ -IC cells are distinct from  $\beta$ -IC cells in that they lack  $Cl^-$  entry steps in the apical membrane. Because IC-cells in the early portion of the rabbit CCD are mainly  $\beta$ -IC cells (22, 23), we did not test the effect of ACh on the  $\alpha$ -IC cell in the present study. We identified the  $\beta$ -IC cell by the following criteria: (a) the apical membrane is stained by FITC-peanut lectin; (b) the resting  $V_B$  is shallow, being less than  $-30$  mV; and (c) a reduction of luminal  $Cl^-$  concentration causes marked hyperpolarization of the  $V_B$ . ACh greatly affected the electrophysiological parameters in the CD cells, but not in the  $\beta$ -IC cells (Table I). The  $\beta$ -IC cells are also found in the CNT and are distinguishable from the CNT cells electrophysiologically (22, 23). Bath application of ACh did not change  $V_T$  and  $R_T$  in the CNT. Taken together, we conclude that ACh has no effect on the ionic conductance of the  $\beta$ -IC cells and that the target cell of ACh is the CD cells in the rabbit CCD.

**ACh inhibits the apical  $Na^+$  channel in the CD cell.** In the present study, we demonstrated that ACh caused a positive  $V_T$



**Figure 8.** Effects of phorbol esters on  $V_T$  and  $R_T$  in the rabbit CCD. Representative tracings are shown in *A*. Note that, in the presence of either PMA (*Aa*) or PDBu (*Ab*), ACh did not produce any additional effects. Phorbol esters and ACh were added in the bath during the period indicated by solid bars in this figure. Summarized data are shown in *B*. The data are expressed as mean  $\pm$  SE. Numbers of observations are shown in *B*. \* $P$  < 0.05; \*\* $P$  < 0.01; \*\*\* $P$  < 0.005, compared to the left values.

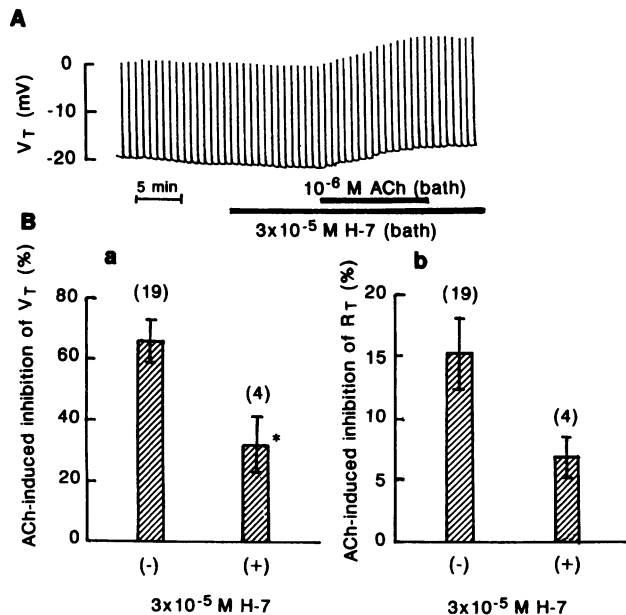
deflection and an increase in  $R_T$ . These effects were closely associated with the actions of ACh in hyperpolarizing the apical membrane and increasing  $fR_A$  of the CD cell. These findings are best explained by the hypothesis that ACh inhibits  $Na^+$

channel in the apical membrane. This hypothesis is further strengthened by the observation that effects of ACh were not observed in the presence of amiloride which blocks  $Na^+$  channel in the apical membrane.

If a  $Cl^-$  channel exists in the apical membrane in the CD cell, its inhibition will lead to similar changes of electrophysiological parameters induced by ACh. At the present, there are no evidences which prove the existence of  $Cl^-$  channel in the apical membrane of the CD cell. Sansom et al. (27) provided evidence for the absence of a significant  $Cl^-$  conductance in the apical membrane of the CD cell. Therefore, it is unlikely that the inhibition of the apical  $Cl^-$  channel accounts for the ACh-induced responses.

Although the CD cell has a high  $K^+$  conductance in the apical membrane (21), it is unlikely that the increase in  $fR_A$  by ACh is due to an inhibition of the  $K^+$  conductance. If ACh inhibited the apical  $K^+$  conductance, the apical membrane would be depolarized rather than hyperpolarized and  $V_T$  would be deflected to a more negative level.

*Muscarinic receptor is involved in the ACh-induced responses.* Several investigators have provided the evidence for the existence of muscarinic receptors in the tubular cells. According to their data, muscarinic receptors were mainly present in the collecting duct cells and were more abundant in the medulla than in the cortex (5, 7). However, little information is available about the characteristics of the receptor type in detail. ACh applied in the bath produced a positive deflection of  $V_T$  and an increase in  $[Ca^{2+}]_i$ . These effects of ACh were antagonized by both atropine and pirenzepine. The antagonistic effect of atropine was tended to be more potent than that of pirenzepine (Figs. 5 and 6). Although CCh also evoked the responses similar to those of ACh, the potency was <10% that of ACh. Our results were compatible with the view of Marchetti et al. (8) deduced from the observations of  $[Ca^{2+}]_i$  in the rat outer medullary CD. The  $M_1$  and  $M_3$  types of muscarinic ACh



**Figure 9.** Effect of H-7 on the ACh-induced changes of  $V_T$  and  $R_T$ . Representative tracings are shown in *A*. Summarized data are shown in *B*. ACh and H-7 were applied to the bathing solution during the period indicated by solid bars in this figure. The ACh-induced inhibition of lumen-negative  $V_T$  (*Ba*) and the ACh-induced increase in  $R_T$  (*Bb*) were compared between the values in the absence and presence of H-7 in the bath, respectively. Each column represents mean  $\pm$  SE. Numbers of observations are shown on the top each column. \* $P$  < 0.05, compared to the control values.

receptors have been shown to couple strongly to phosphatidylinositol turnover. A  $M_1$  receptor has been demonstrated to be highly sensitive to pirenzepine (28, 29). Taken together, it is likely that the muscarinic receptor present in the basolateral membrane in rabbit CD cell is of the  $M_1$  type. However, further investigations are required to determine the exact type of receptor.

**Role of intracellular  $Ca^{2+}$ .** A number of experiments have demonstrated that an increase in  $[Ca^{2+}]_i$  inhibits the  $Na^+$  transport in tight epithelia, such as urinary bladder and ileum (11, 10). Agents which increase  $[Ca^{2+}]_i$ , such as  $PGE_2$  (27, 30, 31), EGF (32–34) and ionomycin (35), were also reported to inhibit the  $Na^+$  transport in the CCD. In the present study, ACh applied in the bath evoked a sustained increase in  $[Ca^{2+}]_i$  and inhibited the apical  $Na^+$  channel. Both the concentration-response relationships of  $V_T$  and  $[Ca^{2+}]_i$  in response to ACh were quite similar. The removal of  $Ca^{2+}$  from the bath, but not from the lumen, partially attenuated the peak amplitude of the ACh-induced rise of  $[Ca^{2+}]_i$  and did completely abolish the sustained component, indicating that ACh increases  $[Ca^{2+}]_i$  via both a release from intracellular store site and an influx across the basolateral membrane. The most parts of the ACh-induced changes of electrophysiological parameters were also attenuated by omission of  $Ca^{2+}$  from the bathing solution, suggesting that for the action of ACh the  $Ca^{2+}$  influx across the basolateral membrane is required. We could not determine whether intracellular free  $Ca^{2+}$  directly or indirectly inhibited the apical  $Na^+$  channel. Palmer and Frindt (36) reported that cytoplasmic  $Ca^{2+}$  had no effect on  $Na^+$  channel in inside-out membrane patch of the rat CCD. Therefore, it is possible that cytoplasmic  $Ca^{2+}$  ion indirectly inhibits  $Na^+$  channel via activation of other signal transduction pathways.

Removal of  $Ca^{2+}$  from the bath, but not from the lumen, caused a significant fall in basal  $[Ca^{2+}]_i$  and a marked attenuation of the ACh-induced sustained increase in  $[Ca^{2+}]_i$ , strongly indicating that  $Ca^{2+}$  permeable pathway exists in the basolateral membrane. Evidence which suggests the existence of  $Ca^{2+}$  entry pathway in the basolateral membrane of the rat CCD has been also demonstrated (37). Recently, we have demonstrated that the application in the bath of manidipine, a dihydropyridine-type of  $Ca^{2+}$  channel blocker, decreases basal  $[Ca^{2+}]_i$  and attenuated the agonist-induced sustained increase in  $[Ca^{2+}]_i$  in the CD cell of the rabbit CCD (18). This would strongly suggest that the dihydropyridine-sensitive  $Ca^{2+}$  channel is present in the basolateral membrane of the CD cell and plays roles in the regulation of both basal  $[Ca^{2+}]_i$  and the agonist-induced increase in  $[Ca^{2+}]_i$ . Therefore, it is possible that ACh evokes a sustained increase in  $[Ca^{2+}]_i$  by the enhancement of  $Ca^{2+}$  influx through the dihydropyridine-sensitive  $Ca^{2+}$  channel in the basolateral membrane. Marchetti et al. (8) reported that  $10 \mu M$  nifedipine failed to block the increase in  $[Ca^{2+}]_i$  caused by CCh and argued that the  $Ca^{2+}$  entry mechanism across the basolateral membrane of the CCD may be distinct from dihydropyridine sensitive  $Ca^{2+}$  channel. Because we did not examine the effect of dihydropyridine in this particular study, the exact nature of the basolateral  $Ca^{2+}$  entry must await further investigations.

**Role of PKC.** It has been demonstrated that  $PGE_2$  inhibits  $Na^+$  channel via an activation of PKC (24). Hays et al. (25) reported that phorbol esters inhibits  $Na^+$  reabsorption and decreases lumen-negative  $V_T$ . Activators of PKC (PMA and 1-oleyl-2-acetyl glycerol) have been shown to inhibit single  $Na^+$

channel in A6 amphibian distal nephron cell line (38, 39). In the  $Ca^{2+}$ -free condition in the bath in our study, ACh evoked only a transient increase in  $[Ca^{2+}]_i$ , indicating that ACh stimulates the production of  $IP_3$  which releases  $Ca^{2+}$  from the internal store site (6). It is possible that an increase in  $IP_3$  production is accompanied in parallel by an increase in diacylglycerol, an endogenous activator of PKC (40). Therefore, we assumed that ACh inhibited the apical  $Na^+$  channel by an activation of PKC. Such assumption is supported by our following findings: (a) both PMA and PDBu inhibited the apical  $Na^+$  channel; (b) in the presence of either PMA or PDBu, ACh produced little or no additional changes of electrophysiological parameters; and (c) H-7 partially attenuated the ACh-induced responses. These results strongly suggest that the activation of PKC in part contributed to the ACh-induced inhibition of the apical  $Na^+$  channel in the rabbit CCD.

**Physiological significance of the ACh-induced inhibition of the apical  $Na^+$  channel in the CCD.** Infusion of ACh into the renal artery have been demonstrated to increase in renal plasma flow, urine volume, and  $Na^+$  excretion without affecting GFR in dogs (1, 2). Both actions of ACh on renal vessels and on tubular reabsorption may account for such phenomena. It has been suggested that the renal actions of ACh were partially mediated by prostaglandins (2). Although other mediators might contribute to the ACh-induced natriuresis, our results strongly suggest that ACh causes natriuresis via an inhibition of  $Na^+$  reabsorption across the CD cells of the CCD.

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