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Research Article

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Effects of Protein Kinase C Activation on Sodium, Potassium, Chloride, and Total CO_2 Transport in the Rabbit Cortical Collecting Tubule

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Abstract

Several hormones induce phosphatidylinositol turnover in cell membranes and thus activate protein kinase C. Activation of protein kinase C can, in turn, have effects on epithelial transport. These experiments were designed to investigate the effects of two activators of protein kinase C, phorbol 12-myristate,13-acetate (PMA) and L-α-1,2-dioctanoylglycerol (L-α-1,2-DOG), and two inactive analogues, 4α-phorbol and 4-*O*-methyl phorbol 12-myristate,13-acetate, on sodium, potassium, chloride, and total CO_2 transport in the rabbit cortical collecting tubule.

Utilizing in vitro microperfusion techniques, we found that activation of protein kinase C with either PMA or L-α-1,2-DOG significantly inhibited net sodium absorption, net potassium secretion and transepithelial voltage in a dose-dependent manner. There was no effect on net chloride or total CO_2 transport. In contrast, the inactive phorbol analogues did not alter either sodium or potassium transport.

These studies demonstrate that in the rabbit cortical collecting tubule sodium and potassium transport can be inhibited by compounds known to activate protein kinase C. Thus, hormones that induce phosphatidylinositol turnover in the rabbit cortical collecting tubule may lead to inhibition of sodium transport by activation of protein kinase C.

Introduction

It is now known that several hormones including vasopressin, bradykinin, and α_1-adrenergics interact with cell membrane surface receptors resulting in phosphatidylinositol (PI)[1] turnover (1, 2). Two intracellular second messengers are formed during PI turnover. The first, inositol-1,4,5-trisphosphate, causes a rise in the cytosolic calcium concentration (3). The second, diacylglycerol, activates a phospholipid and calcium-dependent protein kinase, protein kinase C (4).

Dioctanoylglycerol and the tumor-promoting phorbol esters activate directly protein kinase C, bypassing the initial PI turnover step (5, 6). Schlondorff and Levine have found evidence that protein kinase C activation with phorbol esters can modulate arginine vasopressin (AVP)-stimulated water flow in toad bladder by inhibiting cyclic AMP generation (7). Yanase and Handler have recently demonstrated that activation of protein kinase C with phorbol esters inhibits apical sodium transport of A6 epithelia (8), a cell line that shares many characteristics of the mammalian distal tubule. Thus, it appears that activation of protein kinase C with phorbol esters can modulate sodium and water transport in tight epithelia. Recently, Dixon et al. (9) have detected protein kinase C activity in primary cultures of rabbit collecting tubule cells. The purpose of this study was to examine whether compounds known to activate protein kinase C have an effect on sodium, potassium, chloride, and total CO_2 (bicarbonate) transport in the rabbit cortical collecting tubule (CCT).

Methods

Microperfusion

General experimental features. CCT were dissected and perfused in vitro as previously described (10, 11). Briefly, female New Zealand White rabbits weighing 1.5–2.5 kg were maintained on standard laboratory chow and tap water ad lib. Animals were decapitated and the left kidney rapidly removed, decapsulated, and sliced into 1-mm coronal slices. Slices were placed in an oxygenated bathing solution at 4°C, pH 7.40. The perfusate was an artificial plasma ultrafiltrate containing (in mM) NaCl, 105; KCl, 5; $NaHCO_3$, 25; Na acetate, 10; $NaHPO_4$, 2.3; $CaCl_2$, 1.2; $MgSO_4$, 1; glucose, 8.3; and alanine, 5. The bathing solution was identical in composition except that it contained 2.4 mM $CaCl_2$ and 6 g/100 ml defatted bovine serum albumin (CRG-7; Armour Pharmaceutical Co., Kankakee, IL). Bathing solutions and perfusate were gassed at 37°C with 95% O_2/5% CO_2 to pH 7.35–7.40 and drawn anaerobically into syringes. Cortical collecting tubule segments were identified at the corticomedullary junction and dissected superficially until a branch point was identified. Tubules were cut just distal to the branch point and proximal to the corticomedullary junction for an overall length < 2.0 mm. Tubules were transferred to a thermostatically controlled lucite perfusion chamber and perfused between concentric glass pipettes at 37°C. The tubular ends of each glass holding pipette contained elastomeric silicone resin (Sylgard 184; Dow Corning Corp., Midland, MI) to provide both an electrical and water seal. The perfusion rate was controlled using hydrostatic pressure to a rate of 0.4–2.6 $nl \cdot mm^{-1} \cdot min^{-1}$. Bathing solutions were constantly exchanged at 0.6 ml/min with an infusion pump (Sage Instruments, Division Orion Research, Inc., Cambridge, MA). A perfusion pipette, centered in one of the holding pipettes, was advanced 50–100 μm into the tubule lumen. This pipette also served as an intraluminal electrode because it was connected via a Ringer's agarose bridge to a calomel half-cell. The circuit was completed by a similar agarose bridge placed in the perfusion chamber and connected to a second calomel half-cell. Transepithelial voltage (V_T) thus was monitored continuously

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1. *Abbreviations used in this paper:* AVP, arginine vasopressin; CCT, cortical collecting tubule; CVP, constant volume pipette; L-α-1,2-DOG; L-α-1,2-dioctanoylglycerol; J_V, net volume flux; PMA, phorbol 12-myristate,13-acetate; 4-*O*-methyl PMA, 4-*O*-methyl phorbol 12-myristate,13-acetate; V_T, transepithelial voltage.

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throughout the experiment.[2] V_T was measured with an electrometer (model 602; Keithley Instruments, Inc., Cleveland, OH) and recorded on a strip-chart recorder (Rikadenki model B261; Solter Corp., Sun Valley, CA). Timed samples of tubular fluid were collected in a constant volume pipette (CVP) under water-equilibrated mineral oil. This pipette was calibrated for each experiment as previously described by Schuster et al. (12).

Tubules were equilibrated for 50 min at 37°C before experimentation. Net volume absorption was determined using exhaustively dialyzed methoxy-[^{3}H]inulin (New England Nuclear, Boston, MA) as a volume marker by the method of Schafer et al. (13). Net volume flux (J_v) was calculated from differences in [^{3}H]inulin activity between perfused and collected fluid as previously described (14). Net total CO_2 flux was determined by microcalorimetry. Total CO_2 measurements were made by collecting fluid into 20–30 nl CVPs that were then used to inject the sample into the phosphoric acid chamber of the picapnotherm (Picapnotherm, constructed by the Research and Development Dept., University of California at San Francisco). Each sample was compared to a paired injection of a 25-mM Na_2CO_3 standard and the concentrations of the collected fluid were calculated. Two to four J_{HCO_3} determinations were made with experimental maneuvers. The J_{HCO_3} determinations were averaged for each experimental period and represent a single value for each experiment. J_{TCO_2} is a good estimate of J_{HCO_3} under the conditions studied.

The chloride concentrations of perfusate and collected fluid was measured by electrometric titration using a modification (15) of the technique described by Ramsey et al. (16). All samples were measured at least in triplicate. Two to four collections were made for each period (control, experimental, recovery). The J_{Cl} determinations were averaged for each experimental period and represent a single value for each experiment.

Sodium and potassium content of perfused and collected fluid were measured utilizing flameless atomic absorption spectrophotometry (model video-22; Allied Analytical Systems, Waltham, MA). Each sample was analyzed four to six times and the values averaged. Two to four collections were made for each period. Multiple determinations of standard containing sodium (105–155 mM) and potassium (4–40 mM) in the same range of concentrations as in the perfusate and collected fluid, were performed with each experiment. The values for the sodium and potassium standards were linear over these concentrations with linear regression values of $r = 0.983$ and $r = 0.997$, respectively.

Net bicarbonate, chloride, sodium and potassium fluxes were calculated in picomoles per millimeter per minute as: $J(x) = (V_i (X_i - X_o)/L)$, where $J_{(x)}$ is the net flux of the ion in question; X_i and X_o refers to the concentration of the ion in millimoles per liter in perfusate and collected fluid, respectively; V_i refers to perfusion rate in nanoliters per minute; and L is the tubule length in millimeters. This equation assumes a negligible change in ion concentration due to water movement, which was substantiated by measuring J_v and discarding experiments in which -0.05 nl · mm^{-1} · min$^{-1} \geq J_v \geq 0.05$ nl · mn^{-1} · min^{-1}.

Lumen to bath ^{22}Na flux (J_{Na}^{22} lb) was calculated from measurements of the disappearance of ^{22}Na (40 μCi/ml) from the perfusate. J_{Na}^{22} lb was calculated according to the equation: J_{Na}^{22} lb = [Na]i (V_i/L) ln ([^{22}Na]i/[^{22}Na]o), where [Na]i and [^{22}Na]i are the chemical and isotopic concentrations of sodium in the perfusate; [^{22}Na]o is the isotopic concentration of sodium in the collected fluid, V_i the perfusion rate in nl/min; and L is the tubular length in mm. Samples of methoxy-[^{3}H]inulin and [^{22}Na] were counted in a liquid scintillation counter (Tracor model 6893; TM Analytic, Atlanta, GA) and subsequent experimental values corrected for crossover counting activity of these isotopes. V_T as recorded was the mean value obtained for the entire length of the ion flux measurements for each period and each value represents one experiment.

Experimental protocols. The general protocol was similar in all experiments. After an initial 50-min equilibration period, a control J_v and two to four ion flux determinations were made. The bath was then changed to one containing the test substance. After a 20-min equilibration period, an experimental J_v and two to four ion flux determinations were obtained. In some protocols, a postexperimental period was performed to assess for recovery. During return to control bath, at least a 60-min equilibration period was allotted before J_v and ion flux determinations.

The following experimental protocols were examined

Effect of peritubular phorbol 12-myristate,13-acetate on net lumen to bath ^{22}Na flux (J_{Na}^{22}lb). Control, experimental, and recovery collections were obtained to measure J_v and J_{Na}^{22} lb with perfusate containing 40 μCi [^{22}Na] per ml. The protocol followed is as described. 2.5×10^{-8} M PMA was used in all experiments.

Effect of peritubular phorbol 12-myristate,13-acetate (PMA), L-α-1,2-dioctanoylglycerol (L-α-1,2-DOG), 4α-phorbol, and 4-O-methyl phorbol 12-myristate,13-acetate (4-O-methyl PMA) on net sodium and potassium flux. Control and experimental collections were obtained to measure water, Na^+ and K^+ fluxes as described above. 2.5×10^{-7} M PMA, 2.5×10^{-8} M PMA, 1.6×10^{-9} M PMA, 1.6×10^{-10} M PMA, 7.5×10^{-5} M L-α-1,2-DOG, 5.0×10^{-5} M L-α-1,2-DOG, 5.0×10^{-6} L-α-1,2-DOG, 2.5×10^{-8} M 4α-phorbol, and 1.0×10^{-7} M 4-*O*-methyl PMA were the concentrations of test substances used. An additional period was performed in certain experiments to assess for recovery.

Effect of peritubular PMA on net sodium and potassium flux in the presence of indomethacin. Control, experimental, and recovery collections were obtained to measure water, Na^+ and K^+ fluxes as described above with 5.0×10^{-5} M indomethacin present in the bathing solution during all periods. 2.5×10^{-8} PMA was tested during the experimental period.

Effect of peritubular PMA on net sodium and potassium flux in the presence of protein kinase C inhibition. Collections were obtained to measure water, Na^+ and K^+ fluxes during control conditions and following addition of 7.0×10^{-6} M H-7, a protein kinase C inhibitor (17). An additional period was performed to obtain similar collections in the presence of 7.0×10^{-6} M H-7 and 2.5×10^{-8} M PMA.

Effect of peritubular PMA on net chloride and total CO_2 flux. Control, experimental and recovery collections were obtained as in protocol B, for determining net Cl and total CO_2 fluxes. 2.5×10^{-8} M PMA was used in all experiments.

In all experimental periods involving PMA, 4α-phorbol, and 4-*O*-methyl PMA the glass perfusion syringe was wrapped in aluminum foil and collections obtained utilizing minimal light exposure.

Reagents

PMA, 4α-phorbol, 4-*O*-methyl PMA, and indomethacin were obtained from Sigma Chemical Co. (St. Louis, MO). 4-*O*-Methyl PMA and PMA in chloroform under nitrogen gas were kept frozen for up to 4 wk at −20°C. Solutions of 4α-phorbol in ethanol were maintained in a similar fashion. Aliquots of stock solutions were placed in aluminum foil wrapped flasks and the vehicle evaporated with nitrogen gas before addition of the bathing solution. L-α-1,2-DOG was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL), and kept frozen at −20°C in chloroform under nitrogen gas. Aliquots of this stock solution were dissolved in 100% ethanol after evaporation of the stock with nitrogen gas and the bathing media added for a final ethanol concentration no greater than 0.05%. Concentrations of L-α-1,2-DOG $> 7.5 \times 10^{-5}$ M could not be tested due to limitations of solubility. Control and recovery periods were likewise performed with an ethanol concentration of 0.05%. Indomethacin was prepared as a 0.5 M stock solution dissolved in Na_2CO_3-NaCl mixture as described by Curry et al. (18) to maintain

2. Since bathing and perfusion fluids were symmetric except for bovine serum albumin, the only liquid junction potential that exists is a Donnan potential difference. The maximal Donnan potential difference that may exist is ~ 2 mV. Since this Donnan potential difference was constant during the control, experimental, and recovery periods we did not correct for liquid junction potentials.

Table I. Effects of PMA and L-α-1,2-DOG on Net NA^+ Absorption (J_{Na}), K^+ Secretion (J_K), V_T in the Rabbit CCT

Experimental protocol	n	J_{Na} C	J_{Na} E	J_K C	J_K E	V_T C	V_T E
		$pm \cdot mm^{-1} \cdot min^{-1}$		$pm \cdot mm^{-1} \cdot min^{-1}$		mV	mV
2.5×10^{-7} M Bath PMA	5	26.5±5.8	2.6±0.4	−21.3±2.0	−3.2±0.7	−23.6±3.5	−3.2±0.7
		$P < 0.01$		$P < 0.001$		$P < 0.02$	
2.5×10^{-8} M Bath PMA	9	29.2±5.2	5.3±1.4	−27.8±5.6	−9.4±2.0	−37.1±6.3	−11.0±3.2
		$P < 0.001$		$P < 0.001$		$P < 0.001$	
1.6×10^{-9} M Bath PMA	5	50.5±8.3	33.8±6.1	−28.1±6.0	−19.7±4.1	−48.4±7.8	−29.5±7.7
		$P < 0.005$		$P < 0.05$		$P < 0.001$	
1.6×10^{-10} M Bath PMA	4	50.4±13.9	51.1±14.2	−18.4±3.6	−18.4±4.4	−53.0±8.5	−50.0±10.8
		NS		NS		NS	
7.5×10^{-5} M Bath 1,2-DOG	4	29.9±7.4	19.0±6.2	−18.3±5.3	−10.3±3.2	−32.8±5.7	−16.5±5.9
		$P < 0.005$		$P < 0.02$		$P < 0.001$	
5.0×10^{-5} M Bath 1,2-DOG	6	30.3±5.2	18.1±30	−18.1±2.5	−10.7±1.6	−40.2±9.5	−26.8±5.8
		$P < 0.025$		$P < 0.001$		$P < 0.025$	
5.0×10^{-6} M Bath 1,2-DOG	4	26.9±3.2	24.3±2.8	−23.1±2.8	−19.4±2.6	−31.8±5.1	−28.9±4.6
		NS		NS		NS	

Values are means±SEM.

indomethacin activity. Stock solution was stored as aliquots in the dark at −20°C. H-7 was obtained from Seikagaka America, Inc. (St. Petersburg, FL) and kept at −4°C in distilled water.

Statistics. There were two to four measurements of each parameter in a given period for each tubule. The measurements for individual periods in a given tubule were used to calculate the mean value for that period. Data are expressed as mean±SE. Percentages described in the manuscript are the mean percentage change of each individual tubule. Analysis of variance was used in all experiments where control, experimental, and recovery periods were compared. The two-tailed Student's *t* test for paired and unpaired data were used where appropriate for all other comparisons.

Results

Effects of PMA, L-α-1,2-DOG, 4α-phorbol, and 4-O-methyl PMA on transepithelial voltage. After 60 min of perfusion at 37°C, V_T was stable. The V_T values listed in Table I are the average V_T obtained during the ion flux measurements of control and experimental periods, following the peritubular addition of several different doses of PMA. As shown in Fig. 1, V_T depolarized in a dose-dependent manner with an r value of 0.516 ($P < 0.02$). V_T depolarized significantly by 87.6%, 65.7%, and 43.7% with 2.5×10^{-7} M PMA, 2.5×10^{-8} M PMA and 1.6×10^{-9} M PMA, respectively. 1.6×10^{-10} M PMA did not significantly change V_T. After peritubular addition of 2.5×10^{-8} M PMA, V_T depolarized significantly from control values in 15 tubules (control −38.8±4.0 mV, experimental −13.9±2.5 mV, $P < 0.001$, [Fig. 2]). This depolarization was characterized by two components. An initial rapid depolarization that occurred within 12 min, followed by a more gradual depolarization as long as the tubule was exposed to PMA. Upon return to recovery bath, V_T hyperpolarized significantly to −24.1±3.2 mV ($P < 0.001$). This repolarization was gradual, restabilizing 120–150 min following change to recovery bath.

As we found with peritubular addition of PMA, peritubular addition of both 7.5×10^{-5} M and 5×10^{-5} M L-α-1,2-

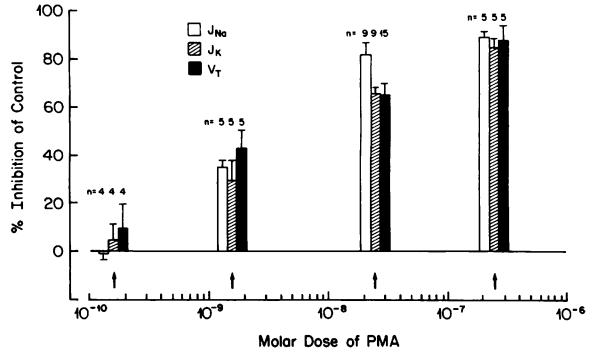

Figure 1. Percent inhibition of control V_T, J_{Na}, and J_K as a function of PMA dosage.

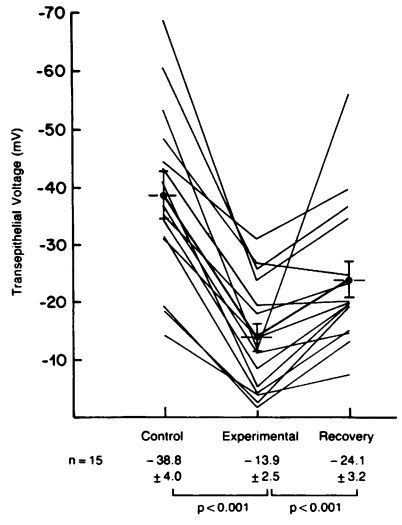

Figure 2. Effect of PMA on cortical collecting tubule V_T. Values for individual tubules during control (*C*), experimental (*E*), and recovery (*R*) periods. Mean V_T±SEM for each period is shown.

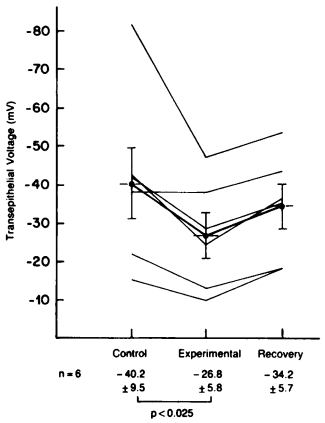

Figure 3. Effect of L-α-1,2-DOG on cortical collecting tubule V_T. See Fig. 2 for symbols and format.

DOG significantly depolarized V_T by 49.7% and 40.2%, respectively (Table I). 5.0×10^{-6} M L-α-1,2-DOG did not significantly depolarize V_T. L-α-1,2-DOG depolarized V_T in a dose-dependent fashion with an r value of 0.714 ($P < 0.005$). Upon removal of the 5×10^{-5} M L-α-1,2-DOG, V_T hyperpolarized to −34.2±5.7 mV (Fig. 3). In contrast to the experiments utilizing PMA, recovery was complete by 20–25 min following withdrawal of peritubular L-α-1,2-DOG. One possibility for the difference in relative recovery rates is that L-α-1,2-DOG unlike PMA is rapidly metabolized by diglyceride lipase within the cell (19). Thus, activation of protein kinase C with two different compounds resulted in an inhibition of V_T.

Shown in Table II are the V_T values obtained during control and experimental periods following peritubular addition of the two inactive phorbol analogues, 4α-phorbol and 4-*O*-methyl PMA. There were no significant differences between control and experimental V_T values with either compound.

Effects of PMA on lumen to bath ^{22}Na flux (J_{Na}^{22} lb). To determine if the effect of PMA on V_T was secondary to a decrease in Na absorption, we first examined the effect of peritubular PMA on J_{Na}^{22} lb. As shown in Fig. 4, peritubular addition of 2.5×10^{-8} M PMA significantly inhibited J_{Na}^{22} lb in five tubules (control 52.2±7.6 pm · mm^{-1} · min^{-1}, experimental 25.7±4.6 pm · min^{-1} · min^{-1}, $P < 0.01$). Upon return to a recovery bath J_{Na}^{22} lb was 30.2±6.3 pm · min^{-1} · min^{-1}. Changes in V_T were similar to those discussed above (control −38.9±3.3 mV, experimental −19.9±4.2 mV, recovery −25.2±3.7 mV).

A time control study demonstrated that J_{Na}^{22} lb remained stable with time (control 61.1±4.2 pm · mm^{-1} · min^{-1}, second control period 61.8±4.2 pm · mm^{-1} · min^{-1}, P = NS, see Fig. 4). V_T was also stable with time (control −36.3±3.7 mV, second control −35.6±5.2 mV, P = NS).

Effects of PMA, L-α-1,2-DOG, 4α-phorbol, and 4-O-methyl PMA on net sodium absorption and net potassium secretion. Since PMA inhibited unidirectional J_{Na}^{22} lb, we next examined the effect of PMA on net Na absorption. PMA inhibited net Na absorption in a dose-dependent manner with an r value of 0.456 ($P < 0.02$, Table I). Following peritubular of addition 2.5×10^{-7} M PMA, 2.5×10^{-8} M PMA, and 1.6×10^{-9} M PMA, net Na absorption significantly fell 88.9%, 82.1%, and 34.6%, respectively (Table I, Figs. 1 and 5). 1.6×10^{-10} M PMA did not significantly alter net Na absorption. In those experiments where peritubular addition of 2.5×10^{-8} M PMA was tested, an additional period was performed to assess for recovery. Following return to control bath, net Na absorption rose to 50.5% of control (Fig. 5). During these experiments, despite a similar inhibition in net Na absorption during the experimental periods, a significant improvement in recovery was found in tubules perfused at faster flow rates ($P < 0.005$). In four tubules perfused at a rate of 2.06±0.31 nl · mm^{-1} · min^{-1} and mean length of 1.98±0.14 mm, recovery was 78.5% of control (control 26.8±2.1 pm · mm^{-1} · min^{-1}, experimental 6.7±2.1 pm · mm^{-1} · min^{-1}, recovery 20.9±2.8 pm · mm^{-1} · min^{-1}). In contrast, in five tubules perfused at a rate of 0.79±0.10 nl · mm^{-1} · min^{-1} and mean length of 1.88±0.11 mm recovery was only 28.1% of control (control 31.1±9.7 pm · mm^{-1} · min^{-1}, experimental 4.2±2.0 pm · mm^{-1} · min^{-1}, recovery 6.9±1.8 pm · mm^{-1} · min^{-1}). The reason for the differences in recovery is not clear, but cannot be explained on the basis of exposure time to PMA, since it was equal in both groups by design.

To determine whether the transport of other ions is altered by the PMA, we next examined the effects of peritubular PMA on net K secretion. Similar to Na, PMA inhibited net K secretion in a dose-dependent manner with an r value of 0.651 ($P < 0.001$). 2.5×10^{-7} M PMA, 2.5×10^{-8} M PMA, and 1.6×10^{-9} M PMA significantly inhibited net K secretion by 85.1%, 66.1%, and 29.6%, respectively (Table I, Figs. 1 and 6), 1.6×10^{-10} M PMA did not significantly alter net K secretion. In those experiments where peritubular addition of 2.5×10^{-8} M PMA was tested an additional period was performed to assess for recovery. There was no significant recovery of net K secretion following return to control bath.

Table II. Effects of 4α-Phorbol and 4-O-Methyl PMA on Net Na^+ Absorption (J_{Na}), K^+ Secretion (J_K) and V_T in Rabbit CCT

		J_{Na}		J_K		V_T	
Protocol	n	C	E	C	E	C	E
		pm · mm^{-1} · min^{-1}		pm · mm^{-1} · min^{-1}		mV	mV
4α-Phorbol	4	46.8±4.4	45.1±5.6	−25.5±1.0	−24.0±1.4	−34.7±4.9	−32.4±3.8
		NS		NS		NS	
4-*O*-Methyl PMA	4	31.4±11.0	34.0±11.1	−26.3±9.2	−24.5±9.4	−32.1±6.0	−30.8±5.8
		NS		NS		NS	
Combined	8	39.1±6.2	39.5±6.1	−25.9±4.3	−24.3±4.4	−33.4±3.6	−31.6±3.2
		NS		NS		NS	

Values are means±SEM.

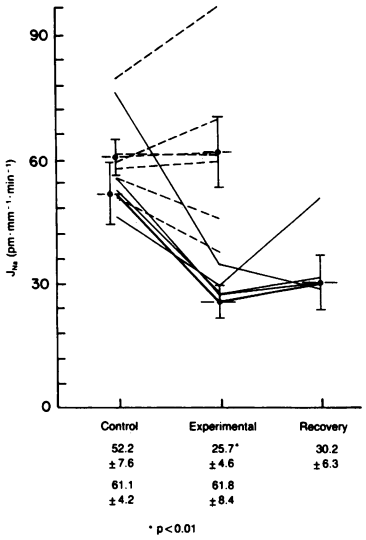

Figure 4. Effect of PMA on cortical collecting tubule lumen to bath ^{22}Na flux (J_{Na}^{22} lb). (——) PMA, $n = 5$ (-----) time control, $n = 6$. $P < 0.01$ control vs. experimental.

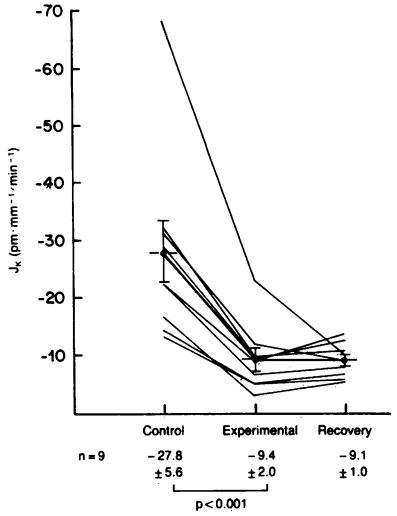

Figure 6. Effect of PMA on cortical collecting tubule net K^+ secretion (J_K).

To confirm that activation of protein kinase C results in an inhibition of both net Na absorption and K secretion in the rabbit CCT, we next determined the effects of another activator of protein kinase C, L-α-1,2-DOG. After peritubular addition of 7.5×10^{-5} M and 5.0×10^{-5} M L-α-1,2-DOG, net Na absorption significantly fell (Table I, Fig. 7). Peritubular addition of 5.0×10^{-6} M L-α-1,2-DOG had no significant effect on net Na absorption. Upon removal of the 5.0×10^{-5} M L-α-1,2-DOG, net Na absorption significantly rose to near control values (control 30.3±5.2 pm · mm^{-1} · min^{-1} vs. 27.4±4.2 pm · mm^{-1} · min^{-1}) (Fig. 7).

Simultaneously, net K secretion significantly decreased 41.4% and 39.2% following peritubular addition of 7.5×10^{-5} M and 5.0×10^{-5} M L-α-1,2-DOG, respectively (Table I, Fig. 8). 5.0×10^{-6} M L-α-1,2-DOG did not significantly inhibit net K secretion (Table I). Upon removal of the 5.0×10^{-5} M L-α-1,2-DOG, net K secretion rose to 77.6% of control values. Thus, activation of protein kinase C with two different compounds resulted in an inhibition of net Na absorption and K secretion.

To ascertain if our findings were due to the nonspecific effect of the phorbol chemical structure, we also examined the effects of peritubular additions of 2.5×10^{-8} M 4α-phorbol and 1.0×10^{-7} M 4-*O*-methyl PMA, two inactive phorbol analogues that do not activate protein kinase C. In four CCTs, peritubular addition of 2.5×10^{-8} M 4α-phorbol had no significant effect on net Na absorption (control 46.8±4.4 pm · mm^{-1} · min^{-1}, experimental 45.1±5.6 pm · mm^{-1} · min^{-1}) or net K secretion (control −25.5±1.0 pm · mm^{-1} · min^{-1}, experimental −24.0±1.4 pm · mm^{-1} · min^{-1}, see Table II). Similarly, peritubular addition of 1.0×10^{-7} M 4-*O*-methyl PMA did not significantly alter net Na absorption (control 31.4±11.0 pm · mm^{-1} · min^{-1}, experimental 34.0±11.1 pm · mm^{-1} · min^{-1}) or net K secretion (control −26.3±9.2± · mm^{-1} · min^{-1}, experimental −24.5±9.4 pm · mm^{-1} · min−1, see Table II) in four CCTs. Thus, compounds with similar chemical structures that do not activate protein kinase C, do not significantly alter net Na absorption, net K secretion, or V_T in the rabbit CCT at similar or higher dosages.

Effect of PMA on net sodium absorption, net potassium secretion and transepithelial voltage in the presence of indomethacin. Because PMA exposure has been associated with an increased production of PGE_2 in both canine kidney (MDCK) cells (20) and toad bladder epithelia (7), and exogenous PGE_2 decreases net Na absorption in the rabbit CCT (21), we next examined if the effects of PMA we found on rabbit CCT net

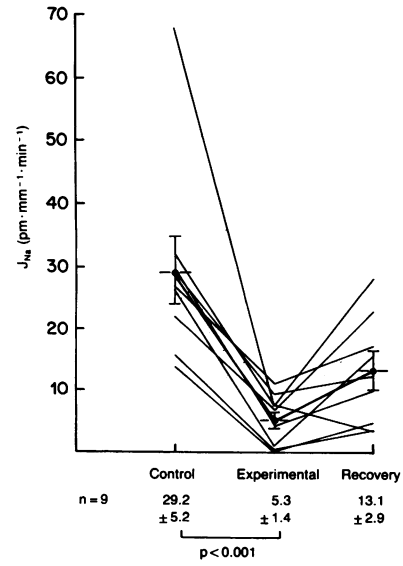

Figure 5. Effect of PMA on cortical collecting tubule net Na^+ absorption (J_{Na}).

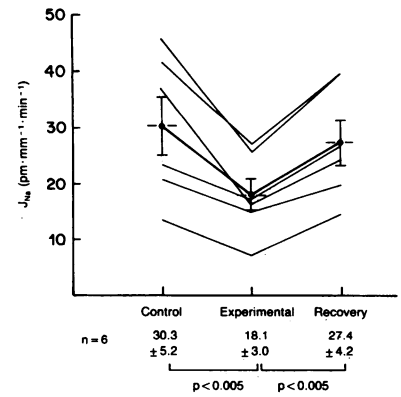

Figure 7. Effect of L-α-1,2-DOG on cortical collecting tubule net Na^+ absorption (J_{Na}).

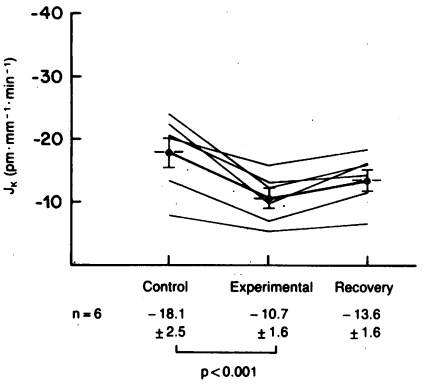

Figure 8. Effect of L-α-1,2-DOG on cortical collecting tubule net K^+ secretion (J_K).

Na absorption and K secretion were secondary to increased endogenous PGE_2 production. These experiments were performed as described above, except for the presence of 5×10^{-5} M peritubular indomethacin in all periods. Previously, Holt and Lechene (22) have found that suppression of endogenous PGE_2 production in the rabbit CCT with 10^{-5} M meclofenamate does not significantly alter V_T, net Na absorption or net K secretion. Moreover, Schuster (23) found no significant effect of 10^{-5} M indomethacin on V_T or J_{Na} ^{22}lb flux in the rabbit CCT. Therefore, indomethacin alone does not appear to alter Na absorption, K secretion or V_T in the rabbit CCT.

In the constant presence of 5×10^{-5} M indomethacin, peritubular addition of 2.5×10^{-8} M PMA still significantly decreased net Na absorption in five tubules (control 22.0±3.4 pm·mm^{-1}·min^{-1}, experimental 8.1±3.2 pm·mm^{-1}·min^{-1} [$P < 0.005$], see Table III). Likewise, net Na absorption significantly rose after return to control bath to 16.7±3.9 pm·mm^{-1}·min^{-1}, ($P < 0.005$). Peritubular addition of 2.5×10^{-8} M PMA also significantly decreased net K secretion in these same tubules (control −16.2±1.9 pm·mm^{-1}·min^{-1}, experimental −6.6±2.3 pm·mm^{-1}·min^{-1}, $P < 0.001$, see Table III). Net K secretion rose but not significantly during return to the control bath to −8.3±1.8 pm·mm^{-1}·min^{-1}. V_T significantly decreased from −28.5±4.9 mV to −4.2±1.5 mV, ($P < 0.005$) following peritubular addition of 2.5×10^{-8} M PMA. V_T significantly hyperpolarized upon return to recovery bath to −18.6±5.1 mV, ($P < 0.005$).

The results obtained in the constant presence of 5×10^{-5} M bath indomethacin were similar to those experiments without indomethacin present. There were no statistical differences between these two groups during the control periods, the degree of inhibition produced by PMA, or the extent of recovery from PMA. Thus, the effects of activation of protein kinase C on V_T, net Na absorption and K secretion are independent from any effect PMA may have on endogenous PGE_2 production.

Effect of PMA on net sodium absorption, net potassium secretion, and transepithelial voltage in the presence of protein kinase C inhibition. To further confirm that activation of protein kinase C results in an inhibition of J_{Na}, J_K, and V_T in the rabbit CCT, we next examined the effect of 2.5×10^{-8} M PMA in the presence of 7.0×10^{-6} M H-7 (a dose predicted to inhibit 54% of protein kinase C activity [17]). Peritubular 7.0×10^{-6} M H-7 produced no significant effect on J_{Na} or V_T, but produced a small but significant fall in J_K (see Table IV). When 2.5×10^{-8} M PMA was added in the presence of H-7, the percent inhibition of J_{Na}, J_K, and V_T was significantly less than when PMA was added alone (see Tables IV and V). The fact that we did not totally inhibit the PMA effect with H-7 would be expected since 7.0×10^{-6} M H-7 is predicted to inhibit only 54% of the protein kinase C activity. These data provide further evidence that activation of protein kinase C results in an inhibition of J_{Na}, J_K, and V_T in the rabbit CCT.

Effects of peritubular PMA on net total CO_2 and chloride transport. We next examined the effect of peritubular PMA on the net transport of total CO_2 in the rabbit CCT. These studies are important because activation of protein kinase C stimulates bicarbonate secretion in Vero cells (from African green monkey kidney [24]), and chicken and rabbit small intestine (25).

There was no significant effect of peritubular addition of 2.5×10^{-8} M PMA on net total CO_2 secretion in eight rabbit CCTs (control −1.56±0.76 pm·min^{-1}·min^{-1}, experimental −0.05±0.55 pm·mm^{-1}·min^{-1}, recovery −0.04±0.56 pm·mm^{-1}·min^{-1}, see Table VI) despite similar changes in V_T that were discussed above (control −32.6±5.6 mV, experimental −5.8±1.4 mV, recovery −8.0±2.1 mV).

Finally, we examined the effects of peritubular PMA on net chloride transport in the rabbit CCT since phorbol esters stimulate Cl secretion in rabbit (25) and rat (26) small intestine and rat colon (27). There was no significant effect of peritubular addition of 2.5×10^{-8} M PMA on net Cl absorption in six rabbit CCTs. Net Cl absorption was 1.16±0.79 pm·mm^{-1}·min^{-1}, 1.81±0.48 pm·mm^{-1}·min^{-1}, and 1.32±0.42 pm·mm^{-1}·min^{-1} during the control, experimental, and recovery periods, respectively (see Table VI). Again, these results

Table III. Effect of PMA on Net Na^+ Absorption (J_{Na}), K^+ Secretion (J_K) and V_T in Rabbit CCT in the Presence or Absence of Indomethacin

		J_{Na}			J_K			V_T		
Protocol	*n*	C	E	R	C	E	R	C	E	R
		pm·mm^{-1}·min^{-1}			*pm·mm^{-1}·min^{-1}*			*mV*	*mV*	*mV*
PMA (in the presence of indomethacin)	5	22.0±3.4	8.1±3.2	16.7±3.9	−16.2±1.9	−6.6±2.3	−8.3±1.8	−28.5±4.9	−4.2±1.5	−18.6±5.1
			$P < 0.005$	$P < 0.005$		$P < 0.001$	NS		$P < 0.005$	$P < 0.005$
PMA alone	9	29.2±5.2	5.3±1.4	13.1±2.9	−27.8±5.6	−9.4±2.0	−9.1±1.0	−37.1±6.3	−11.0±3.2	−20.0±3.2
			$P < 0.001$	NS		$P < 0.001$	NS		$P < 0.025$	$P < 0.025$

Values are means±SEM.

Table IV. Effect of PMA on Net Na^+ Absorption (J_{Na}), K^+ Secretion (J_K) and V_T in Rabbit CCT in the Presence of Protein Kinase C Inhibition (H-7)

Protocol	n	J_{Na} C	J_{Na} H-7	J_{Na} H-7 + PMA	J_K C	J_K H-7	J_K H-7 + PMA	V_T C	V_T H-7	V_T H-7 + PMA
		$pm \cdot mm^{-1} \cdot min^{-1}$			$pm \cdot mm^{-1} \cdot min^{-1}$			mV	mV	mV
2.5×10^{-8} M PMA + 7.0×10^{-6} M H-7	4	40.4±5.7	35.4±6.0	17.9±4.3	−25.5±4.3	−20.5±4.4	−11.3±2.3	−40.9±4.4	−36.4±4.7	−19.5±4.6
			NS	$P<0.001$		$P<0.05$	$P<0.005$		NS	$P<0.001$

	n	J_{Na} C	J_{Na} PMA	J_K C	J_K PMA	V_T C	V_T PMA
2.5×10^{-8} M PMA alone	9	29.2±5.2	5.3±1.4	−27.8±5.6	−9.4±2.0	−37.1±6.3	−11.0±3.2
		$P<0.001$		$P<0.001$		$P<0.025$	

Values are means±SEM.

were obtained despite similar changes in V_T that we discussed above (control −36.9±6.4 mV, experimental −5.4±1.8 mV, recovery −8.3±2.9 mV).

Discussion

The purpose of these studies was to determine whether activation of protein kinase C has an effect on Na, K, Cl, or total CO_2 transport in the rabbit CCT. We found that activation of protein kinase C with PMA or L-α-1,2-DOG significantly inhibited net Na absorption and K secretion in the rabbit CCT, without an effect on net Cl or total CO_2 transport.

Receptor-mediated turnover of PI represents a multifunctional second messenger transducing system (1, 2). The two products of PI turnover, diacylglycerol and inositol-1,4,5-trisphosphate result in activation of protein kinase C and release of cytosolic calcium from endoplasmic reticulum stores, respectively (3, 4). The activation of protein kinase C and its subsequent effects on cells have been investigated utilizing phorbol esters and diacylglycerols (1, 2). Activation of protein kinase C has been shown to alter the transport of Na (8, 27–35), K (36–38), Cl (24–27), HCO_3 (24, 25), and water (7) in different transporting epithelia. Several hormones, neurotransmitters, and growth factors induce PI turnover and thus activation of protein kinase C (1, 2). For example, vasopressin (V_1) and bradykinin induce PI turnover in cell membranes and alter salt or water transport in the rabbit CCT (1, 2, 12, 22, 23).

We have shown that PMA and L-α-1,2-DOG inhibit J_{Na}, J_K, and V_T in a dose-dependent manner. The concentrations of PMA used in our experiments correlate closely with the concentrations of PMA used by Dixon et al. (9) to translocate cytosolic protein kinase C activity to particulate protein kinase C activity in cultures of rabbit CCT cells. Activation of protein kinase C is associated with translocation from cytosolic to particulate fractions (39, 40). Dixon et al. (9) found 35% of the protein kinase C activity to be present in the particulate fraction under control conditions. Following addition of 10^{-10} M and 10^{-8} M PMA, Dixon et al. (9) found an increase in particulate protein kinase C activity to 57% and 93%, respectively. Similarly, 2.5×10^{-5} M 1-oleyl-2-acetylglycerol increased particulate protein kinase C activity to 81%. Thus, the concentrations of PMA used in our experiments correlate closely with the concentrations used by Dixon et al. (9) to activate protein kinase C in the cultured rabbit CCT. Moreover, Hammerman et al. (41) found the K_d of phorbol esters (phorbol 12,13-dibutyrate, PMA) to be 6.2×10^{-8} M in canine cortical basolateral membranes. Therefore, the concentrations of PMA used in our experiments also correlate closely with the concentrations demonstrated by Hammerman et al. (41) for specific binding to renal tissues.

The inhibition of J_{Na}, J_K, and V_T in our experiments appears to be a result of protein kinase C activation. Both PMA and L-α-1,2-DOG, two structurally dissimilar compounds, produce similar results. 4-*O*-Methyl PMA and 4α-phorbol, two compounds structurally similar to PMA that do not activate protein kinase C, do not significantly effect J_{Na}, J_K, or V_T, at concentrations of 1.0×10^{-7} M and 2.5×10^{-8} M, respectively. Moreover, 7.0×10^{-6} M H-7 (a concentration that inhibits ~ 50% of protein kinase C activity [17]) significantly blunts the inhibition of J_{Na}, J_K, and V_T produced by PMA.

Table V. Percent Inhibition of PMA on Net Na^+ Absorption (J_{Na}), K^+ Secretion (J_K), and V_T in Rabbit CCT in the Presence of Protein Kinase C Inhibition (H-7)

Protocol	n	% Inhibition J_{Na}	% Inhibition of J_K	% Inhibition of V_T
A 7.0×10^{-6} M H-7 plus 2.5×10^{-8} M PMA	4	50.4±6.0	44.4±2.2	46.2±3.5
B 2.5×10^{-8} M PMA	9	82.1±5.0	66.1±2.2	65.7±4.6
A vs. B		($P < 0.002$)	($P < 0.001$)	($P < 0.005$)

Values are means±SEM.

Table VI. Effect of PMA on Net Cl Absorption (J_{Cl}), Net Total CO_2 Transport (J_{TCO_2}) in Rabbit CCT

		J_{Cl}			J_{TCO_2}			V_T		
Protocol	*n*	C	E	R	C	E	R	C	E	R
		$pm \cdot mm^{-1} \cdot min^{-1}$			$pm \cdot mm^{-1} \cdot min^{-1}$			*mV*	*mV*	*mV*
PMA	6	1.16±0.79	1.81±0.48	1.32±0.42				−36.9±6.4	−5.4±1.8	−8.3±2.9
								$P < 0.005$		
PMA	8				−1.56±0.76	−0.05±0.55	−0.04±0.56	−32.7±5.6	−5.8±1.5	−8.0±2.1
								$P < 0.005$		

Values are means±SEM.

Recently, Yanase and Handler have found that protein kinase C activation inhibits apical sodium conductance in A6 epithelia (8), a cell line that shows many characteristics of the mammalian collecting duct (42). In our studies, we have found that protein kinase C activation inhibits both lumen to bath ^{22}Na movement as well as net Na absorption in the rabbit CCT, an epithelium that also contains an apical membrane Na conductive pathway (43). Although an inhibition of apical Na conductance by PMA was not tested in out experiments, the time course for activation of protein kinase C and its effects on inhibiting short circuit current in A6 epithelia and inhibiting transepithelial voltage in the rabbit CCT were similar. Thus, it is possible that activation of protein kinase C has a similar effect on inhibiting the apical Na conductance of both A6 epithelia and the rabbit CCT. There is precedence for phorbol esters to affect both apical sodium conductance (8, 31) and Na-K ATPase turnover (29, 34, 35, 44). Both would result in a decrease in Na^+ absorption and K^+ secretion as seen in our studies. The mechanism whereby activation of protein kinase C in the rabbit CCT inhibits net Na absorption remains to be determined.

Phorbol esters such as PMA enhance PGE_2 production in MDCK cells (20) and toad bladder epithelia (7). These findings are an important consideration in our studies since Stokes and Kokko have found that exogenous PGE_2 inhibits net Na absorption and V_T in the rabbit CCT (21). However, our studies performed in the presence of indomethacin argue against enhanced PGE_2 production as a cause for the decreased net Na absorption and V_T that we have found following peritubular application of PMA.

Besides an inhibition in net Na absorption and V_T, we also observed a significant reduction in net K secretion. Both apical K permeability and the electrochemical driving force acting on K ions are important in controlling K secretion in the rabbit CCT. Previously, phorbol esters have been found to block calcium-dependent K conductance in rat hippocampus pyramidal neurons (37), and inhibit K currents evoked by adenosine and cyclic AMP in Xenopus oocytes (38). In the rabbit CCT, when the apical K conductance is inhibited by luminal barium without a change in the electrochemical driving force, Koeppen et al. found V_T not to be significantly altered (43). Thus, the changes in V_T that we observed must be related to the change in sodium transport. The inhibition in K secretion could be secondary to the altered V_T, or could represent a direct effect on the apical K conductance or the basolateral Na-K ATPase.

Previously, the serosal addition of phorbol esters to rabbit and rat small intestine has been found to stimulate Cl secretion in the crypt cell (25, 26), a cell with an apical Cl conductance (26). However, in the rabbit CCT, which has little or no apical Cl conductance (45), we found no evidence for increased net Cl secretion after activation of protein kinase C. Chloride is absorbed in the rabbit CCT by at least two mechanisms (15, 45, 46). Chloride can be absorbed via an apical chloride-bicarbonate exhanger (46), or through the paracellular pathway driven by the lumen-negative voltage that exists secondary to active Na transport (15, 45, 47). In our studies where V_T was inhibited, one might expect choride absorption to be decreased by the latter of the two above mechanisms. However, net chloride absorption was low during our control periods, and we found no significant decrease in net chloride absorption following peritubular addition of PMA. Net K secretion was approximately equal to the net Na absorption in our experiments. Thus, it is likely that the low net Cl absorption rates present in our experiments were secondary to a high net K secretion relative to net Na absorption.

Phorbol esters when applied to Vero cells (from African green monkey kidney [24]) and chicken and rabbit small intestine (25) stimulate bicarbonate secretion. CCTs dissected from normal rabbits have a marked variability in bicarbonate transport with the capacity to either absorb or secrete bicarbonate (48). Therefore, the rabbit CCT can exhibit substantial bidirectional transport of bicarbonate. Our studies contained CCTs with both net bicarbonate secretion and net bicarbonate absorption during the control periods. Neither the CCTs with net bicarbonate absorption or net bicarbonate secretion consistently responded in one direction. Thus, we found no significant effect on net bicarbonate transport. It should be pointed out that the net transport of both total CO_2 and chloride were small during our control periods. It is possible that we were unable to detect changes in net total CO_2 and net chloride transport for this reason. However, the major effect of protein kinase C activation in our studies appears to be on net Na absorption and net K secretion.

In summary, we have found that activation of protein kinase C by peritubular addition of PMA or L-α-1,2-DOG in the rabbit CCT significantly inhibits net Na absorption and K secretion. These effects are independent of endogenous PGE_2 production. Hormones that induce PI turnover may alter sodium transport in the rabbit CCT by activation of protein kinase C. Alternatively, the effect of hormones on the CCT may be modulated by protein kinase C activation.

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