Anti sense DNA down-regulates proteins kinase C-epsilon and enhances vasopressin-stimulated Na+ absorption in rabbit cortical collecting duct.

D L DeCoy, … , J R Snapper, M D Breyer

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Research Article

Hormonal activation of protein kinase C (PKC) is a major signaling mechanism regulating salt and water transport in the distal nephron. We used antisense DNA to down-regulate a PKC isoform in the rabbit cortical collecting duct (CCD) and examined its role in mediating arginine vasopressin's (AVP) effect on salt transport in the CCD. Immunoblots demonstrate that PKC-epsilon (diacylglycerol sensitive) and PKC-zeta (diacylglycerol insensitive) are the major PKC isoforms in both freshly isolated and primary cultures of rabbit CCDs. Rabbit CCDs grown on semi-permeable supports, displayed a positive baseline short circuit current (Isc), which was abolished by amiloride, demonstrating active Na+ absorption. Both AVP and 8-chloro-phenylthio-cAMP (8CPTcAMP) transiently increased Isc, however, within 40 min Isc fell below baseline. Down-regulation of PKC-epsilon, as confirmed by immunoblot, was achieved either by treatment with a PKC-epsilon-specific antisense oligonucleotide or 48 h of 1 microM PMA. In PKC-epsilon down-regulated cells, 8CPTcAMP produced a sustained, rather than transient, increase in Isc. We suggest cAMP stimulates Na+ transport, but secondary activation of PKC-epsilon results in the sustained inhibition of Na+ transport seen in response to vasopressin in the CCD.

Anti sense DNA Down-regulates Protein Kinase C-ϵ and Enhances Vasopressin-stimulated Na^+ Absorption in Rabbit Cortical Collecting Duct

Donald L. DeCoy, James R. Snapper, and Matthew D. Breyer
Departments of Medicine and Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee 37232

Abstract

Hormonal activation of protein kinase C (PKC) is a major signaling mechanism regulating salt and water transport in the distal nephron. We used antisense DNA to down-regulate a PKC isoform in the rabbit cortical collecting duct (CCD) and examined its role in mediating arginine vasopressin's (AVP) effect on salt transport in the CCD. Immunoblots demonstrate that PKC-ϵ (diacylglycerol sensitive) and PKC-ζ (diacylglycerol insensitive) are the major PKC isoforms in both freshly isolated and primary cultures of rabbit CCDs. Rabbit CCDs grown on semi-permeable supports, displayed a positive baseline short circuit current (I_{sc}), which was abolished by amiloride, demonstrating active Na^+ absorption. Both AVP and 8-chloro-phenylthio-cAMP (8CPTcAMP) transiently increased I_{sc}, however within 40 min I_{sc} fell below baseline. Down-regulation of PKC-ϵ, as confirmed by immunoblot, was achieved either by treatment with a PKC-ϵ-specific antisense oligonucleotide or 48 h of 1 μM PMA. In PKC-ϵ down-regulated cells, 8CPTcAMP produced a sustained, rather than transient, increase in I_{sc}. We suggest cAMP stimulates Na^+ transport, but secondary activation of PKC-ϵ results in the sustained inhibition of Na^+ transport seen in response to vasopressin in the CCD. (*J. Clin. Invest.* 1995. 95:2749–2756.) Key words: cyclic AMP • phorbol myristate acetate • electrophysiology • oligonucleotides • kidney

Introduction

The collecting duct is a major site of hormonally regulated Na^+ absorption, and is a major target for the actions of aldosterone, vasopressin, and PGE_2, which contribute to the control of net Na^+ balance. Phosphatidylinositol bisphosphosphate (PIP_2)[1] hydrolysis constitutes a major signaling mechanism by which these hormones control salt and water transport in the CCD (1, 2). The cellular mechanism(s) by which PIP_2 hydrolysis regulates transport are only partially understood. Following phospholipase-C (PLC)–mediated PIP_2 breakdown, the formation of diacylglycerol results in the subsequent activation of protein kinase C (PKC). Numerous studies demonstrate that exogenous PKC activators (such as PMA or diacylglycerols) potently inhibit both Na^+ and water absorption in the cortical collecting duct (CCD) (3, 4). The downstream targets of activated PKC remain less clear.

In the past several years, it has been recognized that the PKCs constitute a multi-gene family of at least 11 different related proteins (5). These can be separated into the Ca^{2+}/diacylglycerol sensitive forms ($\alpha, \beta_1, \beta_2, \gamma$); the Ca^{2+} insensitive, but diacylglycerol-sensitive forms (δ, ϵ, η), and the diacylglycerol insensitive (atypical) forms (ζ and ι). In addition a novel PKC-μ, which is related to ζ, has recently been reported (6). While it is clear that PKC activation is critical for mediating the inhibitory effects of several hormones and autacoids, including PGE_2, EGF, endothelin, and muscarinic agonists, on salt and water absorption in the collecting duct, the PKC isoforms present in the collecting duct remain uncharacterized (2, 7). Studies have also suggested a role for PKC activation in mediating some of the effects of arginine vasopressin (AVP) (8), and PLC-activating V_1 receptors appear to be present in collecting duct (9, 10).

It is generally accepted that the stimulatory effects of vasopressin on CCD Na^+ and water transport can be attributed to V_2 receptor stimulation of cAMP generation activation of protein kinase A, rather than V_1 receptors (2). Both vasopressin and cAMP enhance Na^+ absorption in the CCD (11–15). Interestingly, in the rabbit CCD this effect is only transient (15) and is followed by a sustained period of Na^+ transport inhibition (15, 16). The inhibitory phase appears to depend, in part, on cAMP stimulated Ca^{2+} influx (14). Since PKC may also be activated by increased cell Ca^{2+}, PKC could contribute to the inhibitory effect of cAMP on Na^+ transport (1, 2). Cyclic AMP has been demonstrated to indirectly activate PKC in epithelial cells (17, 18). The purpose of the present studies was to characterize which PKC isoforms are present in the rabbit CCD, and to determine their functional role in mediating vasopressin action in the CCD.

Methods

Collecting duct cell culture. Female New Zealand white rabbits (1–2 kg) were anesthetized with intramuscular ketamine and xylazine (44 and 10 mg/kg, respectively), and killed by decapitation. Both kidneys were perfused with Krebs Ringers and harvested. Rabbit CCD cells were immunodissected as previously described (19). The renal cortex was separated from the capsule and medulla via gross dissection and passed through a tissue press. The dispersed tissue was digested with collagenase (0.1%), hyaluronidase (0.1%), DNase (100 U/ml), and soybean trypsin inhibitor (1000 U/ml, 37°C) in Krebs Ringers. This

Address correspondence to Donald L. DeCoy, F427 ACRE Building, Veterans Administration Medical Center, Nashville, TN 37212. Tel.: (615) 327-4751; FAX: (615) 343-7156.

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1. *Abbreviations used in this paper:* 8CPTcAMP, 8-chlorophenylthio-cyclic AMP; AVP, arginine vasopressin; BCA, bicinchoninic acid; CCD, cortical collecting duct; DAG, diacylglycerol; EVOM, epithelial volt ohmmeter; G_t, transepithelial conductance; I_{sc}, short-circuit current; PIP_2, phosphatidylinositol; PKC, protein kinase C; PLC, phospholipase-C; R_t, transepithelial resistance; V_t, transepithelial voltage.

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suspension was then poured over plates precoated with monoclonal antibody specific for rabbit CCD (3G10) as previously described (19).

Kidney cortex cells were allowed to adhere to sterile polystyrene 100 × 15 mm plastic culture dishes precoated with 3G10 antibody (150 mg of purified monoclonal antibody in 5 ml PBS for 14 h, 4°C) for 10 min. Nonadherent cells were removed by gentle aspiration. The adherent CCD cells were then knocked off the plates with a sharp mechanical blow. Cells were plated onto 4.52 cm^2 collagen-coated semipermeable supports (Costar Corp., Cambridge, MA) at seeding densities of 1.5–2.5 × 10^6 cells per well. Cells were grown to confluence in DMEM (no. 430-2800EB; GIBCO BRL, Gaithersburg, MD) plus 2 grams/liter solution of $NaHCO_3$, 1 μM aldosterone, and 1% Pen-Strep-Neomycin, supplemented with 10% heat-inactivated FBS, at 37°C in a humidified 5% CO_2/21% O_2 atmosphere. Culture medium was replaced every 48 hours. Confluence was assessed both by microscopic inspection and by measurements of transepithelial voltage (Vt) and resistance (Rt) using an epithelial volt-ohmmeter (EVOM; World Precision Instrs., Sarasota, FL; see below). Experiments were run 24–48 h after the final media exchange after confluence was attained.

In some cases, cultured CCDs were treated with antisense or sense oligodeoxyphosphonucleotides (20 μM) or PMA (1 μM) in both the apical and basolateral compartments for 24–48 h before experimentation. In the case of PMA, 1 μM PMA was also added directly to the basolateral side of the Ussing chamber at the beginning of the corresponding short-circuit current experiment. In antisense experiments CCDs were cultured in 0.5% Nu-Serum (Collaborative Biomedical Products, Bedford, MA) (versus 10% FBS) since preliminary studies determined oligonucleotide stability was significantly enhanced by these culture conditions.

Oligonucleotide design and synthesis. The antisense and sense unmodified oligodeoxyphosphonucleotides to the PKC isoforms were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). The sequences were 18 base pairs long with the sense sequence being 5′-ATG-GTA-GTG-TTC-AAT-GGC-3′ and the antisense 5′-GCC-ATT-GAA-CAC-TAC-CAT-3′. The sequences (sense and antisense) were comprised of the start codon (ATG) plus the 15 additional, downstream base pairs in the rabbit PKC-ϵ sequence (20). This region was chosen since it appears to be uniquely effective in down-regulating protein translation (21). The product was dissolved in water to make a final stock solution of 10^{-3} or 10^{-2} M and kept frozen at −4°C until ready to use.

Oligonucleotide stability. To assess stability of oligonucleotides in culture media, antisense to the PKC-ϵ oligomer was 5′ end labeled with $\gamma^{32}P$ and added to the cultured cells grown in 10, 5, or 0.5% FBS and 0.5% Nu-Serum at a concentration of 8 nM in the presence of an excess of unlabeled carrier oligomer, to achieve a final concentration of 4 μM. As a control the same amount of labeled oligomer was added to media without cells. 3-μl aliquots were taken at 0, 2, 4, 8, 12, and 24 h from each medium and mixed with 7 μl of sequencing buffer and then loaded onto a 20% acrylamide gel. After electrophoresis the was gel exposed to x-ray film and oligomer degradation assessed as per Holt et al. (22).

Measurements of culture media electrolytes. After confluence, 1-ml samples of media were collected from the apical and basolateral sides either at the time of experimentation or 48 h after a media exchange. Na^+, K^+, Cl^-, and HCO_3^-, determinations were made using ion sensitive microelectrodes (Synchron model CX-7; Beckman Instrs., Carlsbad, CA) in the Clinical Chemistry Laboratory (VA Medical Center, Nashville, TN). Medium pH was measured using a pH/blood gas analyzer (model 1302; Instrumentation Laboratory Systems, Lexington, MA).

Electrophysiology. Electrophysiologic studies were performed using both an EVOM to study cells in the tissue culture incubator, as well as studying the cells in an Ussing chamber (Jim's Instrument's, Iowa City, IA). For studies using the EVOM, the polarity of the V_t readings are given using the basolateral compartment as reference. Rabbit CCDs cultured on 4.52 cm^2 Transwell inserts, reached confluence 6–7 d after plating. Transmembrane resistance increased from < 400 Ω-cm^2 to > 1,500–2,000 Ω-cm^2 as confluence was achieved. Transepithelial voltage typically exceeded −20 mV (basolateral reference) after confluence. Both V_t and R_t reached plateau values after 7–10 d in culture, and were stable for approximately an additional week. Only CCD monolayers exhibiting apical negative voltages of −20 to −80 mV (as measured by the EVOM) with resistance of at least 1,000 Ω-cm^2 were used for experimentation. After the Transwells® were transferred to the Ussing chamber, V_t and R_t were ~ 30% lower than that measured by the EVOM immediately before transfer. I_{sc} also showed a slow decline over the first 20–30 min in the chamber and either stabilized or continued to slightly decline. A similar fall in V_t and R_t was noted in the culture dish (as measured by EVOM) when fresh media was added to the Transwells® at the routine 48-h interval, even without mounting the transwells in the Ussing chamber. The recovery of V_t or R_t to their prior levels usually took 12–18 h. This has been noted previously by other investigators (23) although the cause is unknown.

The Ussing chamber was specifically designed to house 4.52-cm^2 Costar transwells. Each half of the Ussing chamber had solution reservoirs of ~ 19 ml that were filled with DMEM/HCO_3 medium with an osmolality of ~ 300 (mOsms/kg H_2O) that was continuously bubbled with 95% O_2/5% CO_2, yielding a pH of 7.30–7.40. The temperature was maintained at 37°C using a recirculating heated water bath and a YSI thermistor to continuously determine the temperature.

Each half chamber was connected to a current clamp amplifier (model VCC600; Physiological Instruments, San Diego, CA) by two agarose/KCl electrodes (3% agar/1 M KCl), one to measure V_t and the other to pass current. The agarose bridges were connected to the amplifier via a 1 M KCl reservoir and a Ag-AgCl electrode connected to the amplifier head-stage device. Open-circuit V_t, transepithelial conductance (G_t, mS/cm^2) and I_{sc} (μA/cm^2) were determined. I_{sc} was measured when V_t was clamped to 0 mV. Transepithelial conductance G_t (and its inverse, resistance R_t) was measured from the current deflection (I) in response to a 10 mV offset lasting 3 s, and obtained every 90 s, using Ohm's Law and the relationship $G_t = I/V_t$. The clamp was zeroed before each experiment by inserting a blank transwell as described above and placing sterile Ringer's solution into each half-chamber. After transferring the cells into the Ussing chamber, ~ 20–40 min equilibration was allowed for stabilization of I_{sc} and G_t. Individual experiments lasted 2–4 h. AVP, 8CPTcAMP, and PMA were always added to the basolateral half-chamber, while amiloride was added to the apical compartment.

Protein content determination for PAGE. Protein loading for each lane of PAGE was normalized by a bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL). The protein concentration for each unknown CCD cell homogenate was determined from a standard curve of varying BSA concentrations. This procedure showed a protein concentration of ~ 1.80 μg/μl, following which 0.5 ml of SDS-PAGE sample buffer was added to the apical compartment of each Transwell (as described below) to further dilute the concentration to 1.20 μg/μl. 25 μl of cellular extract in SDS-PAGE buffer was pipetted onto each lane of the polyacrylamide gel for a final mass of 30 μg protein/lane.

Immunologic characterization of PKC isoforms in CCDs. Freshly immunodissected CCDs, or CCDs grown to confluence on 4.52 cm^2 transwells as described above, were studied. For cultured cells, four confluent wells were washed with Kreb's Ringers solution three times. This was followed by the addition of 0.5 ml of SDS-PAGE sample buffer to the apical compartment followed by repetitive aspiration. This material was then transferred to a 1.5-ml conical tube and 20 μl of 5% β-mercaptoethanol was added, followed by heating in boiling water for 3 min. The extract (25 μl/lane) was loaded onto a Bio-Rad precast SDS-PAGE 4–15% gradient mini-gel and run at 100 V until the dye front had run off, except in the case of detecting for PKC-ζ in which the gel was run for an additional 20 min after the dye front disappeared. The proteins were transferred to nitrocellulose paper at 200 V for 2.5 h. The nitrocellulose strips were then stored at −20°C until ready for immunostaining.

Nitrocellulose paper was washed three times with blocking buffer (Tris-buffered saline which contained 150 mM NaCl, 50 mM Tris, 0.05% Tween 20 detergent, and 5% Carnation nonfat dry milk, pH 7.5)

Table I. Ion Concentrations and pH of Apical and Basolateral Culture Media of CCD Cells at 48 h

	Baseline	48 h Apical	48 h Basolateral
		mmol/l	
Sodium	136	110*	156
Potassium	5.8	18.6*	3.4
Chloride	119	117	123
pH	7.35	6.15*	7.11

Results are means of 28 samples with baseline values measured from stock solution of prepared medium of DMEM/HCO_3^- with 10% fetal bovine serum and 1 μM aldosterone. Samples were taken at 48 h after medium was exchanged in mature monolayers. * $P < 0.05$ apical vs. basolateral by paired *t*-test.

for 1 h at room temperature. This was followed by three washings with blocking buffer at 5-min intervals. The nitrocellulose strips were then incubated in primary antibody (isoform-specific rabbit polyclonal PKC antibodies; GIBCO BRL) diluted 1:300 in blocking buffer for 1 h at room temperature. Following three additional washings, the strips were incubated with biotinylated, anti-rabbit IgG (Vectastain Kit) for 1 h, followed by three 5-min washings. Antibody labeling was visualized using Avidin-DH coupled to horseradish peroxidase (Vector Laboratories, Inc., Burlingame, CA) followed by addition of diaminobenzidine and hydrogen peroxide (in 20 ml 100 mM Tris-saline) plus 100 μl of 8% $NiCl_2$ (to enhance visualization of the reaction). The blots were incubated for 5–10 min with this mixture to obtain the desired colorimetric intensity.

Statistics. Short-circuit current, transepithelial voltage, and transepithelial conductance were compared by one way ANOVA or paired/unpaired-*t* test where appropriate. $P < 0.05$ was considered statistically significant.

Reagents. AVP, 8CPTcAMP, PMA and other reagents were purchased from Sigma unless stated otherwise.

Results

Baseline electrophysiology and transport characteristics. Confluent CCDs developed pH and electrolyte gradients within 48 h of replenishment of apical and basolateral medium (Table I). Apical compartment Na^+ concentration decreased and apical compartment K^+ concentration increased, consistent with known transport properties in the CCD (1, 24). The apical compartment was consistently acidic in confluent CCDs. Cl^- concentrations did not significantly change after 48 h.

Effect of vasopressin on I_{sc} in the CCD. Primary cultures of rabbit CCDs, grown on Transwells, were mounted in the Ussing chamber and treated with 230 pM arginine vasopressin added to the basolateral half-chamber. Basal currents ($n = 4$), averaged 9.68±1.52 μA/cm^2 and increased within 2 min or less after AVP administration. This was followed by a broader peak to 14.83±1.60 μA/cm^2 ~ 15 min after the compound was added. R_t simultaneously decreased from 903±75 Ω-cm^2 to 773±56 Ω-cm^2 at the peak current suggesting AVP increased apical to basolateral cation transport rather than decreasing a parallel opposing current.

40 min after AVP addition I_{sc} had dropped below basal values to 6.38±1.26 μA/cm^2 ($P < 0.05$). At the same time R_t increased slightly to 814±72 Ω-cm^2. Addition of 1 μM amiloride to the apical side abruptly decreased I_{sc} to −3.95±1.28 μA/cm^2, suggesting the positive I_{sc} was due to active Na^+ transport (Fig. 1 *A*). All changes in I_{sc} noted above were statistically significant (paired *t*-test or one way ANOVA).

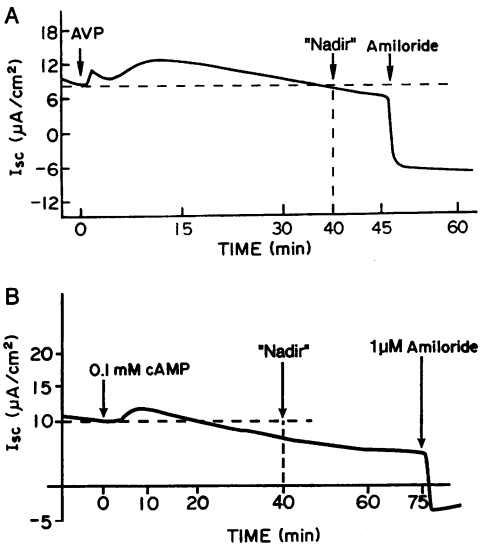

Figure 1. Effect of arginine vasopressin and 8CPTcAMP on short-circuit current (I_{sc}, μA/cm^2) in mature cultured rabbit CCD cells. (*A*) Short-circuit current tracing of confluent CCD cells treated with 230 pM arginine vasopressin in the basolateral half-chamber of an Ussing chamber ($n = 4$). AVP transiently increased I_{sc} above baseline. However, subsequently I_{sc} fell below baseline in the continued presence of AVP. Addition of 1 μM amiloride to the apical half-chamber abruptly reduced I_{sc} below zero in all experiments. (*B*) Short-circuit current in confluent CCDs is stimulated by 0.1 mM 8CPTcAMP, $n = 8$. As with AVP, a biphasic response in I_{sc} was seen, with the initial increase occurring within approximately 20 min. 1 μM apical amiloride addition reduced I_{sc} below zero demonstrating the positive current was due to Na^+ transport from apical to basolateral compartments.

Effect of cAMP on I_{sc} in the CCD. Like AVP, 8CPTcAMP had a biphasic effect on I_{sc} (Fig. 1 *B*). Baseline I_{sc} was 6.62 μA/cm^2±2.5 and transiently increased to 8.5 μA/cm^2±2.5, 10–20 min after addition of 0.1 mM 8CPTcAMP to the basolateral reservoir ($n = 8$, $P < 0.025$, ANOVA) (Table II). Transepithelial resistance simultaneously decreased from 790±6.7 Ω-cm^2 to 685±6.1 Ω-cm^2. I_{sc} then progressively declined below baseline, over the next 20–25 min. 40 min after 8CPTcAMP (defined as "nadir") I_{sc} had fallen by 4.7±2.3 μA/cm^2 below baseline, to 1.9±0.76 μA/cm^2 ($P < 0.05$). Apical amiloride (10^{-6} or 10^{-5} M) caused I_{sc} to fall to −3.4±0.4 μA/cm^2 resulting in a ΔI_{sc} of −4.4±1.2 μA/cm^2 from the nadir, suggesting the pre-amiloride positive current was due to Na^+ transport via amiloride sensitive channels.

Acute effect of PMA on V_t and R_t and I_{sc}. To examine the effect of acute PMA addition on I_{sc}, PMA (1–10 μM) was added to the basolateral reservoir of the Ussing chamber. Addition of 1–10 μM PMA resulted in a slow decline in I_{sc} over 10–15 min (data not shown). However, because of the variable, preceding spontaneous decline in I_{sc} observed after transfer of transwells to the Ussing chamber, these results were difficult to interpret.

We therefore tested the effects of 1 μM PMA in DMSO, or DMSO alone (0.01% wt/vol) on V_t and R_t in CCDs directly in the transwells, using an epithelial volt-ohmmeter. V_t decreased exponentially in PMA-treated cells ($n = 4$) from an initial value

Table II. Effect of 8CPTcAMP on I_{SC} in Control and Chronic PMA-treated CCD Cells

	n	Baseline	cAMP Peak	cAMP "Nadir"
Control	8	6.62±2.5	8.50±2.5*	1.90±0.76§
Chronic PMA	6	12.6±3.2	17.5±3.8‡	16.8±4.0§

Confluent monolayers of CCD cells were placed in an Ussing chamber and, after stabilization of current and resistance, treated with 8CPTcAMP. Chronic PMA treatment is defined as incubation with 1 μM PMA for 48 h. I_{SC} reported in μA/cm² and above values are means±standard error. Nadir values were taken 40 min after cAMP addition to the basolateral reservoir of the chamber. Note that the nadir in the control experiments is well below the baseline current while in the PMA-pretreated cells it remains significantly above baseline. * $P < 0.025$ by ANOVA comparing peak to baseline for control cells, while ‡ $P < 0.01$ by paired *t*-test for the same comparison in chronic PMA-treated cells. § $P < 0.05$ by paired *t*-test for controls and chronic PMA-treated cultures for nadir to baseline values. $P = 0.05$ by unpaired *t*-test for baseline currents in the control and chronic PMA-treated cells.

of −50.1±5.4 mV to −2.1±1.6 mV. This effect was noticed within 30 min with a drop in V_t to −41±4.9 mV. R_t concomitantly increased from 2336±203 Ω-cm² to 4325±528 Ω-cm² 330 min after PMA addition. Control (DMSO-treated) cells showed no change in V_t or R_t over the same time period (Fig. 2). The difference between V_t and R_t in the PMA treated versus DMSO control groups were statistically significant.

Effect of chronic PMA incubation on I_{sc}. Chronic PMA treatment is associated with PKC down-regulation (25). Confluent CCD monolayers were treated with 1 μM PMA (basolateral compartment of the culture dishes) for 48 h. Baseline current averaged +12.6±3.2 μA/cm² ($n = 6$) and increased to

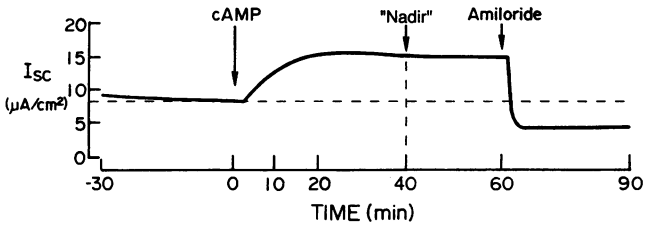

Figure 3. Effect of chronic PMA on cAMP stimulated I_{sc} in cultured rabbit CCDs. Confluent CCDs were incubated for 48 h before experimentation with 1 μM PMA in order to deplete PKC, $n = 6$. Short-circuit current tracing demonstrating a sustained monophasic rise in positive current in response to 0.1 mM 8CPTcAMP. Addition of 1 μM amiloride to the apical half-chamber decreased I_{sc}. However, in contrast to non–PMA-treated cells, amiloride-insensitive I_{sc} remained positive.

+17.5±3.8 μA/cm² within 20 min of addition of 0.1 mM 8CPTcAMP (ΔI_{sc} 4.9 μA/cm²±1.0; $P < 0.01$). This increase in I_{sc} was nearly three times greater than that produced by 8CPTcAMP in non–PMA-treated CCDs (Table II). Furthermore, in PMA-pretreated cells, this increase was sustained, with I_{sc} remaining above baseline for at least 60 min after 8CPTcAMP. The I_{sc} at 40 min after 8CPTcAMP (the arbitrary "nadir") was +16.8±4.0 μA/cm², nearly nine times greater than the current at the same time in control cells.

Chronic PMA treatment enhanced the amiloride-sensitive sodium current as reflected by the threefold increase in the amiloride sensitive component of the I_{sc} (Fig. 3). After the addition of 1 μM amiloride to the apical compartment of the Ussing chamber, I_{sc} decreased by 12.6±3.2 μA/cm² in PKC down-regulated cells versus 4.4±1.0 μA/cm² in control cells ($n = 6$ each).

Effect of sense and antisense PKC-ε oligonucleotides. Pre-

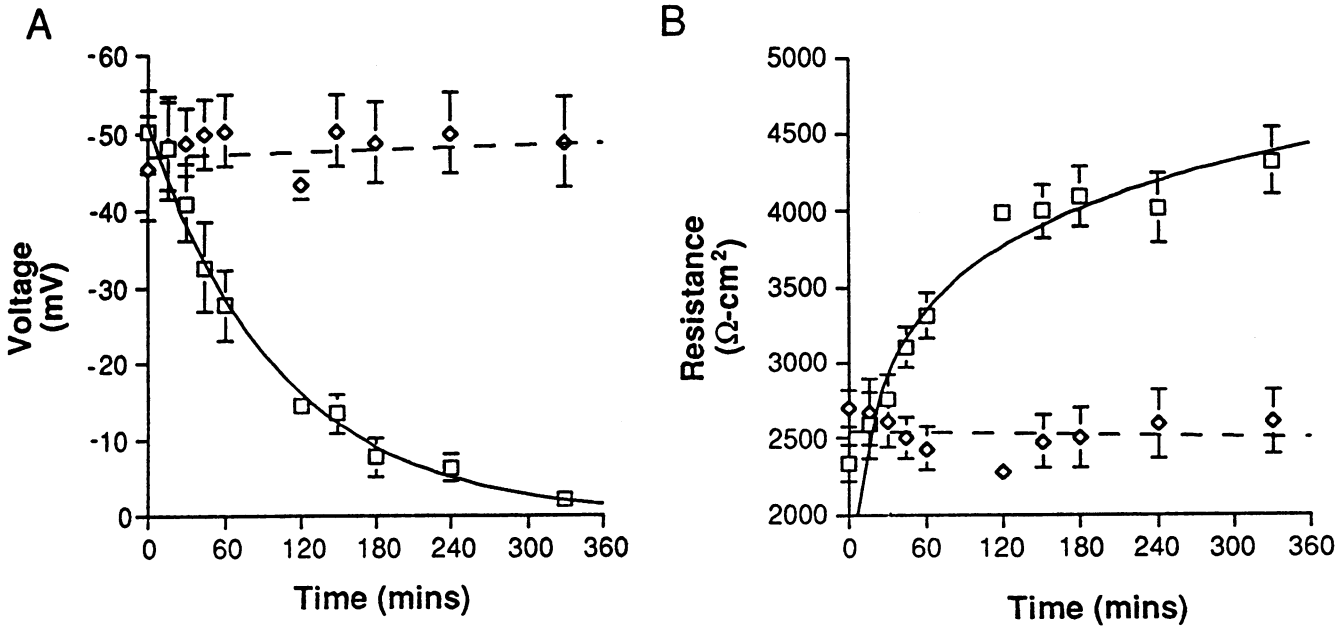

Figure 2. (*A* and *B*) Acute effect of PMA on transepithelial voltage (V_t) and resistance (R_t) in cultured rabbit CCDs. (*A*) Transepithelial voltage (V_t) vs. time and (*B*) R_t vs. time. Cultured CCDs were treated with either 1 μM PMA (*solid line*) or vehicle (0.01% wt/vol DMSO, dashed line). Cells were incubated at 37°C in a humidified 5% CO_2/21% O_2 environment between measurements of V_t and R_t using an EVOM. V_t decreased exponentially after PMA treatment with a concomitant increase in R_t. No change for either V_t or R_t occurred in the DMSO-treated cells. $n = 4$.

Table III. Effect of Antisense Oligonucleotides on I_{SC} in Cultured Rabbit CCDs

	n	Baseline	cAMP Peak	cAMP "Nadir"
Sense	5	8.74±2.30	11.7±2.45*	8.66±2.21
Antisense	6	9.77±2.43	15.02±3.28*	13.52±3.04‡
No Oligo	4	12.10±4.10	15.23±4.79*	9.53±2.69

Short-circuit currents (I_{SC}, $\mu A/cm^2$) of CCD monolayers measured in an Ussing chamber after incubation with either 20 μM sense or antisense oligonucleotides to PKC-ϵ. Once confluence was achieved the media was changed to DMEM/HCO_3^- with 1 μM aldosterone and 0.5% Nu-Serum in order to minimize oligonucleotide breakdown (see Methods). ΔI_{SC} from peak to "Nadir" in the sense oligo-treated cells was 3.63±1.01 $\mu A/cm^2$, while in the antisense-treated cells the value was 1.25±0.24 ($P < 0.05$ by unpaired *t*-test). * $P < 0.05$ by paired *t*-test comparing peak to baseline currents in all three groups, while ‡ $P < 0.01$ for nadir to baseline in the antisense-treated cultures. *P* values for nadir to baseline were not significant in the sense and no oligonucleotide (*control*) treated cells.

liminary experiments showed that 0.5% Nu-Serum rather than 10% FBS was necessary to preserve oligomer integrity for 24 h. Considerable degradation of incubation of ^{32}P-labeled oligomers was observed within 6 h of incubation in culture media using 10 and 5% FBS (data not shown). For this reason the oligomer-treated cells were grown in 0.5% Nu-Serum.

After achieving confluence in 10% FBS, CCDs were switched to 0.5% Nu-Serum. In some experiments 20 μM sense or antisense oligodeoxyphosphonucleotides were added to apical and basolateral compartments for 48 h before study. 8CPTcAMP stimulated I_{sc} in control-, sense-, and antisense-treated cells. However this effect was sustained only in the PKC-ϵ antisense-treated cells (Table III, Fig. 4). 40 min after 8CPTcAMP treatment I_{sc} remained above baseline only in the antisense-treated cells, whereas it fell below baseline in the control- and sense oligomer-treated cells (ΔI_{sc}, Fig. 4). Treatment with 1 μM amiloride completely inhibited the positive current leaving residual currents of −4.4±0.45 $\mu A/cm^2$ and −1.2±0.98 $\mu A/cm^2$ in sense and antisense-treated cells, respectively.

PKC's isoform expression and PKC-ϵ down-regulation in the CCD. Immunoblots of rabbit CCDs using isoform-selective polyclonal anti-PKC antibodies suggest the ϵ isoform (83 kD M_r) and ζ isoforms (~ 68 kD M_r) (5) are the major PKC isoforms present in freshly isolated and cultured rabbit CCDs (Figs. 5–7). In addition, PKC-γ appeared to be present, but only in the cultured cells (Fig. 7). The specificity of the antibodies to PKC-ϵ, γ, and ζ was confirmed by incubation with the peptide to which the each PKC-selective antibody was made (Figs. 6, lane *2* and 7, lane *2*). Specific competition of the appropriate band with the peptide could be demonstrated. In each case we observed nonspecific labeling of at least 2–3 bands migrating at an apparent M_r of 65–70 and 100 kD*a*. Since these were observed in the absence of primary antibody (see Fig. 5, lane 1), they appear to represent nonspecific reactivity of the secondary antibodies.

PKC down-regulation by chronic PMA treatment or antisense DNA was confirmed by immunoblot. Chronic treatment with PMA down-regulated PKC-ϵ but not PKC-ζ as compared with DMSO-treated cells (Fig. 6). This result is consistent with the absence of a phorbol ester binding site on the PKC-ζ isoform and hence its known insensitivity to down-regulation by phorbol esters (5). PKC-ϵ antisense 20–40 μM also resulted in selective down-regulation of the PKC-ϵ band (Fig. 7). Neither the

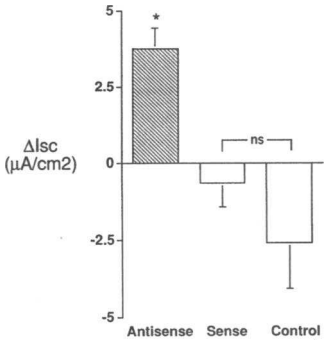

Figure 4. Effect of PKC-ϵ sense and antisense oligonucleotides on ΔI_{sc} after cAMP. The change in short-circuit current (ΔI_{sc}, $\mu A/cm^2$), 40 min after cAMP addition (nadir point) as compared to baseline I_{sc} (just before cAMP) was measured in control, PKC-ϵ sense and antisense oligomer-treated cultured CCDs. The ΔI_{sc} in the antisense-treated cells (*hatched box*) was +3.75±0.69 ($n = 6$), while it was −0.64±0.80 and −2.58±1.50 in the sense-treated ($n = 5$) and control cells ($n = 4$), respectively ($P \leq 0.025$ for antisense vs. sense or control cells). No significant difference was noted between the ΔI_{sc} for sense vs. control cells.

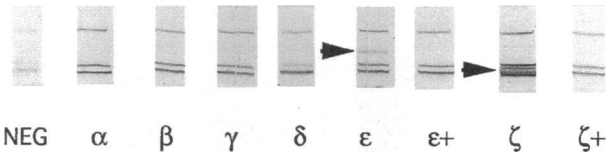

Figure 5. Immunoblots for the PKC isoforms in freshly immunodissected rabbit CCDs. Immunoblots of freshly prepared CCDs demonstrate the presence of only the epsilon and zeta PKC isoforms (*arrowheads*). + designates addition of the corresponding peptide against which the antibody was generated, demonstrating specificity of the antibody for the putative PKC band. No PKC specific band was detected in lanes incubated with the α, β, γ, or δ antibodies.

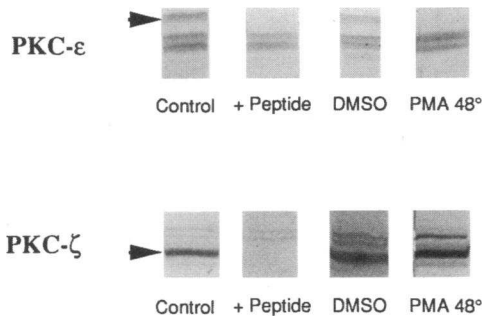

Figure 6. Chronic PMA treatment down-regulates PKC-ϵ in cultured rabbit CCD's. The top panel depicts immunodetection of PKC-ϵ, while PKC-ζ is displayed in the lower panel. Immunostains of mature rabbit CCD cells treated with 1 μM phorbol ester (*PMA*) for 48 h (lane *4*) showed down-regulation of PKC-ϵ and not PKC-ζ. Both the ϵ and ζ isoforms were detected in the non-PMA (*control*) treated cultures (lane *1*). Antibody specificity was confirmed by incubation with the corresponding peptide (lane *2*) in the control cells. Vehicle alone (DMSO, 0.01% wt/vol did not affect either the ϵ or ζ (lane *3*). Before immunostaining for PKC-ζ in the lower panel, SDS-PAGE was performed for an additional 20 min after the dye front had run off to allow better discrimination between the ζ band and the nonspecific bands.

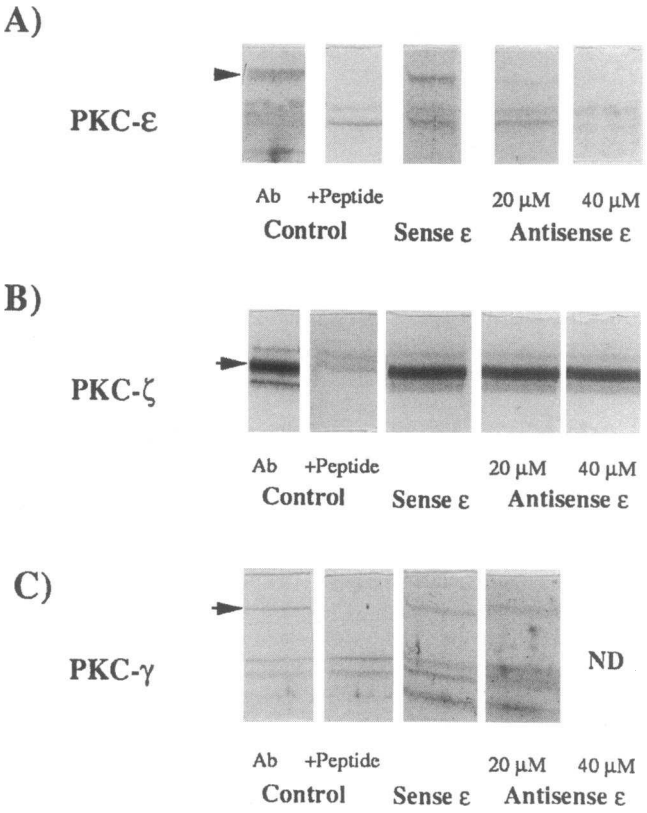

Figure 7. Antisense selectively down-regulates PKC-ϵ in cultured rabbit CCD's. Immunoblots for PKC-ϵ, ζ, and γ isoforms (*A–C*, respectively) in confluent CCDs treated with either 20 μM antisense PKC-ϵ oligonucleotides or 20 μM sense oligonucleotides. The ϵ, ζ, and γ isoforms are seen in the control (no oligo) and sense-treated cells, while 20 μM antisense treatment almost completely abolished PKC-ϵ but had no effect on PKC-ζ or PKC-γ (*middle boxes*). Incubation with 40 μM PKC-ϵ antisense showed complete loss of the ϵ band. Antibody specificity was confirmed by competition of the ϵ, ζ, and γ bands in blots incubated with the corresponding peptide. *ND,* not done.

PKC-γ nor the PKC-ζ bands were affected by PKC-ϵ sense or antisense treatment.

Discussion

The present studies examined the role of PKC in mediating vasopressin action in the CCD (1, 2). It is now appreciated that the PKCs constitute a multigene family, comprised of at least 11 different subtypes (5, 26). While PKC activation is critical for mediating the inhibitory effects of several hormonal agonists on CCD salt and water transport, the identity of the specific PKC isoforms in this nephron segment remains uncharacterized. In the present studies we found only PKC-ϵ and PKC-ζ were detected by immunoblots of freshly immunodissected CCD preparations. PKC-γ was detected in cultured CCDs. It remains unclear whether the expression of PKC-γ in cultured CCDs is due to culture induced dedifferentiation of these cells, or whether small amounts of PKC-γ are also present in freshly isolated CCDs.

We focused on PKC-ϵ and PKC-ζ, because these isoforms are highly expressed in both freshly isolated CCDs and in primary cultures of CCDs. PKC-ϵ is a member of the so called "novel" PKC group (5). The novel PKCs are activated by diacylglycerols and phorbol esters, but unlike the α, β, or γ isoforms, they are not activated by Ca^{2+} and hence are termed Ca^{2+} independent. PKC-ζ is a member of the so-called atypical PKCs and does not possess a diacylglycerol binding site (5, 27). The function and mechanism of PKC-ζ activation is not well understood. Since hormonal activation of PIP_2 hydrolysis is thought to activate PKC through diacylglycerol (DAG) formation, the present observations suggest that PKC-ϵ is the major DAG-sensitive PKC isoform mediating hormone action in the fresh CCD.

The functional role of PKC-ϵ in hormone action in the CCD was examined using two maneuvers to down-regulate PKC-ϵ: chronic treatment of cultured CCDs with phorbol esters and incubation with PKC-ϵ–specific antisense oligonucleotides. Functional studies were performed on cultured CCDs rather than isolated microperfused CCDs to allow PKC down-regulation with these agents for the required 24–48 h. Treatment with the PKC-ϵ antisense 18-nucleotide oligomer, but not the sense oligomer, markedly down-regulated immunodetectable PKC-ϵ. PKC down-regulation was specific for PKC-ϵ, having no effect on PKC-ζ or PKC-γ expression. Similarly, immunodetectable PKC-ϵ was down-regulated by chronic PMA treatment, while PKC-ζ remained unchanged. The latter results are predicted by the absence of a phorbol ester binding site on PKC-ζ, so that chronic PMA treatment should not modify its expression. In contrast to the antisense experiments, chronic PMA treatment would also be predicted to down regulate PKC-γ in cultured CCDs. Since the significance of PKC-γ expression in cultured but not freshly isolated CCDs is unclear, this was not directly confirmed. We examined the effect of PKC-ϵ down-regulation on vasopressin-regulated ion transport in primary cultures of rabbit CCDs.

Cultured CCDs display many characteristics similar to freshly microdissected CCDs (1, 28, 29). Apical V_t was negative and developed a significant basolateral > apical, Na^+ concentration gradient over 48 h consistent with electrogenic Na^+ absorption. The apical medium was also acidified in confluent monolayers by ~ 1.0 pH units as compared to basolateral pH. This acidification may be due to intercalated cells in cultured CCDs (24), and electrogenic H^+ secretion is supported by the presence of an apical-positive voltage and negative I_{sc} post-amiloride (29). Apical K^+ concentration also increased by more than fivefold over the basolateral compartment. Thus, like the microperfused CCD, cultured CCDs actively absorb Na^+ from apical to basolateral compartments, and secrete H^+ and K^+.

Primary cultures of rabbit CCDs also exhibit electrogenic ion transport regulated by vasopressin, similar to that observed in the microperfused CCD. Vasopressin and cAMP produce a biphasic effect. I_{sc} transiently increased and this was followed by a sustained decrease in the flow of positive current occurring within 40 min of AVP or cAMP addition. Similarly AVP and cAMP only transiently stimulate, and then inhibit Na^+ transport in the isolated perfused CCD (13–15). Because meclofenamate attenuated the inhibitory phase in the microperfused CCD, Holt and Lechene (15) suggested that stimulation of endogenous PGE_2 synthesis accounted for this inhibitory effect, however, others were unable to reproduce these results (16). Additional mechanisms including increased Ca^{2+} (14) and PKC activation may contribute to this inhibitory phase.

We examined the role of PKC activation in mediating the inhibitory phase. PKC activators potently inhibit Na^+ absorp-

tion in the microperfused rabbit CCD (3). The present studies show in cultured CCDs, addition of exogenous phorbol esters reduced I_{sc} and V_t, while increasing R_t. This suggests PKC activation inhibits Na^+ absorption in cultured CCDs, as observed in fresh CCDs (3). As discussed above, both PKC-ϵ anti-sense oligomers and phorbol esters potently down-regulated PKC-ϵ in cultured rabbit CCDs. Down-regulation of PKC-ϵ completely abolished the inhibitory phase seen in response to cAMP, converting it to a monophasic increase in I_{sc}. This sustained positive current after cAMP reflects electrogenic Na^+ transport since it was completely abolished by 1 μM amiloride. It is notable that higher baseline currents were observed in the chronic PMA-treated cells. This may reflect a tonic inhibitory effect of the PKCs on Na^+ absorption that is eliminated when PKCs are down-regulated.

In the collecting duct, vasopressin receptors stimulate both cAMP generation (V_2-receptor) and PIP_2 hydrolysis resulting in PKC activation (via the V_1 receptor) (9, 10). However, cAMP completely mimics vasopressin's biphasic effect on Na^+ transport in both microperfused (14) and cultured CCDs. Thus, the inhibitory phase can be accounted for solely by V_2 receptor stimulation of cAMP generation, without invoking V_1 receptor activation. Recent evidence suggests that significant cross-talk between cAMP and PKC pathways exists. Cyclic AMP activates PKC in both airway epithelial and renal epithelial cells (17, 18). The present studies provide evidence that cross-talk between cAMP and PKC pathways may have important functional implications with respect to vasopressin regulation of Na^+ transport in the collecting duct.

Cyclic AMP increases intracellular Ca^{2+} in the microperfused CCD (14), providing a possible mechanism for cAMP dependent PKC activation. Previous work confirms increased intracellular calcium inhibits Na^+ transport by inhibiting the amiloride-sensitive Na^+ channel in the CCD (30) and that this effect is indirect and mediated by some critical cytosolic component (31), possibly PKC (32). However, calcium-activated PKC (either α, β, or γ) do not appear to be highly expressed in freshly isolated CCDs. Immunodetectable PKC-γ was present in cultured CCD's. Whether PKC-γ is also expressed in much lower levels in freshly isolated CCDs will require confirmation using more sensitive techniques. Nevertheless, selective down-regulation of PKC-ϵ appears to be sufficient to prevent the inhibitory effect of cAMP on CCD Na^+ transport (Fig. 4 and 7). Although Ca^{2+}-dependent PKC isoforms may not be highly expressed in freshly isolated CCD, it is still possible that increased $[Ca^{2+}]_i$ could activate Ca^{2+}-insensitive PKC isoforms, specifically PKC-ϵ. Ca^{2+} dependent activation of phospholipase-C or -D would lead to diacylglycerol formation (5) thereby activating PKC-ϵ.

Salt and water transport in the CCD are under control by two, functionally antagonistic, signaling pathways: the stimulatory cAMP system, and the inhibitory PIP_2/PKC system (1, 2). The present studies now provide functional evidence for cross-talk between cAMP activation and PKC activation. The sustained inhibitory effect of cAMP on Na^+ transport is dependent on PKC-ϵ expression. Alterations in PKC-ϵ expression or activity in vivo could significantly modulate the net effect of AVP on whole body Na^+ balance, possibly converting the effect of AVP from being natriuretic (33) to stimulating Na^+ retention (34). An important role for vasopressin in the regulation of Na^+ balance including the development of salt-sensitive hypertension has been suggested (34, 35). Mineralocorticoid-induced hypertension may be associated with enhancement of AVP dependent Na^+ absorption in the CCD (34, 36), however, whether mineralocorticoid action involves down-regulation of PKC activity remains to be determined. Nevertheless, the present studies suggest modulation of PKC expression could significantly alter the renal effect of Na^+-retaining hormones.

In summary these studies show: (*a*) PKC-ϵ and ζ are the major PKC-isoforms expressed in freshly isolated and primary cultures rabbit CCDs, while cultured CCDs additionally express PKC-γ; (*b*) 8CPTcAMP transiently stimulates amiloride-sensitive Na^+ transport from apical to basolateral compartments in the rabbit CCD and this is followed by sustained inhibition of Na^+ transport; (*c*) acute PKC activation with phorbol esters inhibits transport in cultured CCDs; and (*d*) PKC-ϵ down-regulation enhances cAMP stimulated transport and abolishes the secondary inhibitory effect on amiloride-sensitive I_{sc}. These observations suggest a central role for PKC-ϵ in regulating vasopressin action and Na^+ transport in the CCD. We propose that PKC-ϵ activation counter-balances the stimulatory effect of cAMP on amiloride-sensitive Na^+ transport. Factors which reduce PKC-ϵ expression may enhance hormonally stimulated Na^+ absorption in the CCD.

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