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Differential Effects of Aldosterone and Vasopressin on Chloride Fluxes in Transimmortalized Mouse Cortical Collecting Duct Cells

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Abstract. The effects of aldosterone and vasopressin on Cl^- transport were investigated in a mouse cortical collecting duct (mpkCCD) cell line derived from a transgenic mouse carrying the SV40 large T antigen driven by the proximal regulatory sequences of the L-pyruvate kinase gene. The cells had features of a tight epithelium and expressed the amiloride-sensitive sodium channel and the cystic fibrosis transmembrane conductance regulator (CFTR) genes. dD-arginine vasopressin (dDAVP) caused a rapid, dose-dependent, increase in short-circuit current (I_{sc}). Experiments with ion channel blockers and apical ion substitution showed that the current represented amiloride-sensitive Na^+ and 5-nitro-2-(3-phenylpropylamino)benzoate-sensitive and glibenclamide-sensitive Cl^- fluxes. Aldosterone (5×10^{-7}M for 3 or 24 hr) stimulated I_{sc} and apical-to-basal $^{22}Na^+$ flux by 3-fold. $^{36}Cl^-$ flux studies showed that dDAVP and aldosterone stimulated net Cl^- reabsorption and that dDAVP potentiated the action of aldosterone on Cl^- transport. Whereas aldosterone affected only the apical-to-basal $^{36}Cl^-$ flux, dDAVP mainly increased the apical-to-basal Cl^- flux and the basal-to-apical flux of Cl^- to a lesser extent. These results suggest that the discrete dDAVP-elicited Cl^- secretion involves the CFTR and that dDAVP and aldosterone may affect in different ways the observed increased Cl^- reabsorption in this model of mouse cultured cortical collecting duct cells.

Key words: Kidney — Corticosteroid hormones — Vasopressin — Short-circuit current — Sodium channel — Cystic fibrosis transmembrane regulator

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Introduction

The direction of ion transport in polarized epithelial cells depends on whether the specific ion channels, passive and active transporters and cotransporters are apical or basolateral. The net movement of Na^+ and water also results from the coordinated chloride uptake mechanisms coupled to the sodium gradient, and the opening of chloride channels on the other side of the cells (Frizzell, Field & Schultz, 1979). In tight epithelia, such as distal tubules and collecting ducts, the distal colon and the ducts of exocrine glands, the epithelial sodium channel (ENaC) governs the entry of Na^+, which is then extruded by the basolaterally-located Na^+-K^+ ATPase pumps (Rossier & Palmer, 1992; Verrey, 1995).

The kidney collecting duct cells play a key role in the final reabsorption of salt, which is regulated by corticosteroid and antidiuretic hormones (Stokes, 1995). Most of our knowledge of how aldosterone mediates Na^+ transport has come from in vitro studies (Rossier & Palmer, 1992; Verrey, 1995) conducted on the amphibian A6 kidney cells (Handler, Perkins & Johnson, 1981). However, the effects of the antidiuretic hormone of nonmammalian vertebrates, vasotocin, or arginine vasopressin (AVP) on Cl^- transport in A6 cells (Chalfant, Coupaye-Gerard & Kleyman, 1993; Verrey, 1994) or in mammalian collecting duct cells (Canessa & Schafer, 1992; Nagy, Naray-Fejes-Toth, & Fejes-Toth, 1994; Kizer, Lewis & Stanton, 1995) are less well known. This is in part because collecting ducts are composed of principal and intercalated cells, each having different reabsorptive and secretory transport capacities (Schuster, 1995), and partly because there is no consensus as to the Cl^- conductance specifically implicated in Cl^- transport mediated by AVP. AVP is reported to stimulate electrogenic chloride secretion by mouse inner medullary col-

lecting duct (IMCD) cells in culture (Kizer, Lewis & Stanton, 1995), and the cystic fibrosis transmembrane conductance regulator (CFTR) is probably involved in this process (Husted et al., 1995; Vandorpe et al., 1995). In a recent study, Morris et al. (1998) have demonstrated the presence of CFTR in A6 cells and that the vasotocin-stimulated Cl^- current was significantly inhibited by glibenclamide, an inhibitor of CFTR (Sheppard & Welsh, 1992). CFTR is also expressed in the cortical collecting duct (CCD) cells (Todd-Turla et al., 1996), but its participation in Cl^- transport has yet to be proved unambiguously.

Few studies have examined the combined effects of aldosterone and AVP on Cl^- transport by collecting duct cells (Canessa & Schafer, 1992; Kizer, Lewis & Stanton, 1995). These hormones act in synergy to increase NaCl absorption. Verrey (1994) showed that vasotocin caused a rapid increase in transepithelial Cl^- conductance in A6 cells, which was followed by a slower increase in Na^+ transport, and that these effects were potentiated by prolonged incubation with aldosterone. AVP also potentiates the increase in short-circuit current (I_{sc}) induced by long-term incubation (48 hr) of cultured rabbit CCD cells (Canessa & Schafer, 1992) and mouse IMCD cells (Kizer et al., 1995) with aldosterone, but the contribution of net Cl^- movements was not investigated.

The lack of an established mammalian CCD cell line that has retained its sodium transport stimulated by aldosterone has hampered analyses of the short- and long-term effects of aldosterone and AVP on Na^+ and Cl^- transport. There is thus a need for a model of mammalian CCD cells which is suitable for studying the action of aldosterone and AVP on ion transport, and particularly on Cl^- fluxes. The strategy of targeted oncogenesis in transgenic mice, where an oncogene [generally the SV40 large T and little t antigens (Tag)] is under the control of the regulatory sequences of a tissue-specific gene, has been used to produce a variety of differentiated immortalized renal cell lines (Briand, Kahn & Vandewalle, 1995). Recently, Miquerol et al. (1996) generated a line of transgenic mice, SV-PG/Tag mice, carrying Tag under the control of the proximal region (-1000 bp) of the L-type pyruvate kinase (L-PK) gene fused to the SV40 enhancer. mRNA and immunofluorescence studies demonstrated that the transgene was actively expressed in CCD and IMCD cells from mice fed a carbohydrate-rich diet (Miquerol et al., 1996). These results led us to use this strain of transgenic mice to develop immortalized, differentiated CCD cells that could be maintained in vitro.

This report describes the properties of a line of immortalized CCD cells, mpkCCD cells, derived from cCD microdissected out from a one-month old SV-PK/Tag transgenic male mouse. The cells have the main features of the parental CCD, including *ENaC* (Canessa et al., 1994) and *CFTR* (Todd-Turla et al., 1996) gene expressions. I_{sc} experiments with specific ion channel blockers identified an apically located amiloride-sensitive Na^+ conductance and apical and basolateral Cl^- conductances. Deamino-8-D-arginine vasopressin (dDAVP), which binds specifically to the V2 receptors (Kurokawa et al., 1992), increased I_{sc} in a dose-dependent manner. Experiments with apical Na^+- and Cl^--free substituted solutions showed that the rise in I_{sc} induced by dDAVP corresponded to Na^+ and Cl^- fluxes. Aldosterone (5×10^{-7}M) stimulated I_{sc} as well as the apical-to-basal $^{22}Na^+$ flux in a time-dependent manner. dDAVP further increased I_{sc} from cells incubated with aldosterone for 3 and 24 hr. $^{36}Cl^-$ flux studies also showed that dDAVP potentiated the increase in net Cl^- reabsorption induced by aldosterone.

Materials and Methods

Materials

Culture media (DMEM, HAM's F12) were from Life Technologies (Eragny, France). $^{36}Cl^-$ and $^{22}Na^+$ were purchased from Amersham (Les Ulis, France). [α-^{32}P]dCTP was from NEN (Le Blanc Mesnil, France). dDAVP was purchased from Ferring Pharmaceutical (Sweden). Other hormones and reagents were from Sigma (St. Louis, MO). The rabbit anti-Tag anti-rabbit polyclonal antibody was a gift from Dr. Hanahan (San Francisco, CA). The anti-cytokeratin K_8-K_{18} antibody was kindly provided by Dr. D. Paulin (Institut Pasteur, Paris VII, France). The anti-ZO-1 antibody was from Cedarlan Laboratories. NPPB was purchased from ICN Pharmaceuticals (Natick, MA). The RNA-PLUS extraction kit was purchased from Bioprobe systems (Montreuil-sous-bois, France). The Moloney murine leukemia virus (MMLV) reverse transcriptase was from Life Technologies. Tissue Culture Treated Transwell or Snapwell filters (0.4 μm pore size, 1.2 cm^2 diameter) were from Corning Costar Corp. (Cambridge, MA). The Millicell Electrical Resistance clamp apparatus (VCC 600) was from Precision Instrument Design (Tahoe City, CA).

Cell Culture

Studies were carried out on mpkCCD cells derived from isolated CCD tubules microdissected out from the kidney of a SV-PK/Tag transgenic mouse carrying the SV40 large T and little t antigens under the control of the SV40 enhancer placed in front of the -1000 bp fragment of the rat L-L′-PK gene regulatory region in the 5′ flanking region (Miquerol et al., 1996). mpkCCD cells were established using the protocol described earlier for mouse proximal tubule and intestinal crypt cell lines (Lacave et al., 1993; Bens et al., 1996). Kidneys were removed and thin slices of cortex were incubated in DMEM: HAM's F12 (vol/vol) containing 0.1% (w/vol) collagenase for one hour at 37°C. The CCD fragments were rinsed in medium without enzyme and microdissected. Pools of 5–10 isolated CCD were transferred to collagen-coated 24-well trays, and cultured in a defined medium [DM: DMEM: HAM's F12, 1:1 vol/vol: 60 nM sodium selenate; 5 μg/ml transferrin; 2 mM glutamine; 50 nM dexamethasone; 1 nM triiodothyronine; 10 nM epidermal growth factor (EGF); 5 μg/ml insulin; 20 mM D-glucose; 2% fetal calf serum (FCS); 20 mM HEPES, pH 7.4] at 37°C in 5% CO_2-95% air atmosphere. They were passaged in 12- and 6-well trays, and

then routinely grown in 25 cm^2 culture flasks at 37°C in a 5% CO_2-95% air atmosphere. Medium was changed every two days and all studies were performed on cells between the 15th and 35th passages that had been grown on plastic Petri dishes, glass slides, or semi-permeable filters.

Morphological and Immunohistochemical Studies

Confluent cells grown on 60-mm plastic Petri dishes were examined under an inverted microscope (Zeiss) equipped with phase-contrast optics. Indirect immunofluorescence studies were performed on confluent cells fixed with 60% acetone-30% ethanol or ice-cold methanol. Tag was immunodetected using a specific anti-rabbit polyclonal antibody (Bens et al., 1996). Cells were also processed for immunofluorescence using anti-cytokeratin K_8-K_{18} and anti-ZO-1 antibodies (Bens et al., 1996). All preparations were examined under a Zeiss photomicroscope equipped with epifluorescence optics. For electron microscopy, confluent cells grown on filters were fixed for 2 hr with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, embedded in Epon, and processed for transmission electron microscopy.

RNA Extraction and Reverse Transcriptase/Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from confluent cells grown on filters using the RNA-PLUS extraction kit. RNA (2 μg) was reverse transcribed with MMLV reverse transcriptase at 42°C for 45 min, and 50–100 ng cDNA and nonreverse-transcribed RNA were amplified for 25–28 cycles in 100 μl total volume containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 40 μM dNTP, 1.5 mM $MgCl_2$, 1μCi [α-^{32}P]dCTP, 1 unit Taq polymerase, 29.2, 31.5 or 29.4 pmol of α-, β-, γ-rENaC primers, respectively, and 9 pmol of hGAPDH (internal standard) primers. The same procedure was followed using 31 pmol of CFTR primers and 0.2 pmol of β-actin (internal standard) primers. The sets of rENaC and hGAPDH primers used were the same as the ones described by Hummler et al. (1996). The two CFTR primers were MCF6 (5′-ATGAGGTTCTTCACTAGCTCT-3′) in the mouse CFTR exon 7 and MCF8 (5′-CCTCCCAAAATGCTGTTACAT-3′) in the mouse CFTR exon 8 (Bens et al., 1996). β-actin primers (5′-CGTGGGCCGCCC-TAGGCACCA-3′ and 5′-TTGGCCTTAGGGTTCAGGGGGG-3′) were the same as previously described (Bens et al., 1996). The thermal cycling programs were as follows: 94°C for 30 sec, 55°C (CFTR), 54°C (α-rENaC) or 53°C (β- and γ-rENaC) for 30 sec, and 72°C for 60 sec. Amplification products were run on 4% polyacrylamide gels and autoradiographed.

cAMP Content

Cell cyclic AMP (cAMP) was assayed as described (Lacave et al., 1993). Confluent cells grown on 12-well trays were incubated without or with dDAVP (10^{-6}M), parathormone (1–34 synthetic fragment from bovine parathormone, PTH: 10^{-7}M), isoproterenol (ISO: 10^{-6}M) or calcitonin (CT: 100 ng/ml) for 7 min at 37°C. The reaction was stopped by rapidly removing the medium and adding 1-ml ice-cold 95% ethanol-5% acid formic solution. The supernatants were evaporated to dryness and cAMP was determined using the Pasteur radioimmunoassay kit (n° 79830, Institut Pasteur, France).

Electrophysiological Studies

Cells were grown on collagen-coated Transwell filters in DM medium until confluency (day 6) and then in DM without EGF, hormones and FCS (HFM; the HEPES was replaced by 15 mM $NaHCO_3$) for a final 18 hr. Cells for experiments with aldosterone were cultivated in HFM supplemented with charcoal-treated (steroid-free) FCS for 24 hr and then in $NaHCO_3$-HFM medium for 18 hr. Transepithelial electrical resistance (R_T) and voltage (V_T) were measured using dual silver/silver chloride (Ag/AgCl) electrodes connected to the Millicell Electrical Resistance System (ERS, Millipore corporation, Bedford, MA). Equivalent I_{sc} was calculated with Ohm's law from R_T and V_T. Snapwell filters were mounted in a Ussing-type chamber (Diffusion Chamber System, Costar Cambridge, MA) for direct I_{sc} measurements, and the chamber connected to a voltage clamp apparatus via glass barrel Micro-Reference Ag/AgCl electrodes filled with 3M KCl. Cell layers were bathed on each side with 8 ml HFM medium prewarmed to 37°C and continuously gassed with 95% O_2–5% CO_2 to keep the pH at 7.4. I_{sc} (μA/cm^2) was measured by clamping the open-circuit V_T to 0 mV for 1 sec. By convention, positive I_{sc} corresponded to a flow of positive charges from the apical to the basal solution. Under these conditions, R_T was calculated with Ohm's law from V_T and I_{sc}. The transepithelial conductance (G_T) is $1/R_T$.

Apical Ion Substitution Experiments

I_{sc} was measured as described above on sets of confluent cells in which the $NaHCO_3$-HFM medium from the apical side was replaced by Na^+-free (*N*-methyl-D-glucamine, 156 mM; KCl, 4 mM; $MgCl_2$, 0.7 mM; $MgSO_4$, 0.4 mM; $CaCl_2$, 1.05 mM; glucose, 20 mM; HEPES, 8 mM) or Cl^--free (gluconate, 127 mM; Na_2HPO_4, 1 mM; $NaHCO_3$, 30 mM; $HKCO_3$, 4.2 mM; $CaSO_4$, 1.05 mM; glucose, 20 mM; HEPES, 8 mM) solutions. The basal side of the filter was always bathed with the HFM medium (NaCl, 120 mM; KCl, 4.2 mM; $NaHCO_3$, 34 mM; $MgCl_2$, 0.3 mM; Na_2HPO_4, 1 mM; $MgSO_4$, 0.4 mM; $CaCl_2$, 1.05 mM; glucose, 20 mM). I_{sc} was measured after a one-hour equilibration period in both Na- and Cl-free conditions.

$^{22}Na^+$ and $^{36}Cl^-$-Flux Studies

The transepithelial transport of $^{22}Na^+$ and $^{36}Cl^-$ (50 nCi/ml) from the apical-to-basal and from the basal-to-apical sides was measured on confluent cells grown on filters. The inside of the filters was filled with 700 μl and the outside with 1.2 ml. For apical-to-basal flux measurements 50 nCi/ml $^{22}Na^+$ or $^{36}Cl^-$ were added to the HFM bathing the apical (inside) side of the cells, and 50 nCi/ml $^{36}Cl^-$ was added to the HFM bathing the basal side (outside) of the cells for basal-to-apical flux measurements. In both cases, monolayers were incubated at 37°C, and 50 μl apical or basal samples were collected 10, 30 and 60 min after adding the radioactive tracers to the opposite side of the filters. The radioactivity was then measured in a liquid scintillation counter (LKB, Pharmacia). The results are expressed as nEq per cm^2 filter area.

Data Analysis

Results are expressed as means ± SE from (*n*) experiments. Significant differences were analyzed by Student's *t*-test and mean comparison for ion flux experiments were performed by one way or two ways analysis of variance followed by the Student-Newman-Keuls method.

Abbreviations

CCD	cortical collecting duct
IMCD	inner medullary collecting duct
L-PK	L-type pyruvate kinase

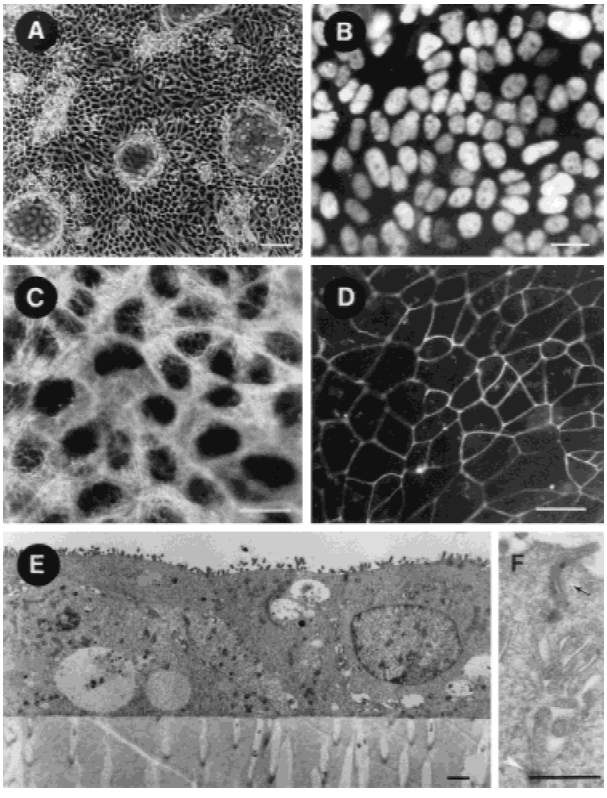

Fig. 1. Morphology of mpkCCD cells. Confluent cells grown on Petri dishes have a uniform ''cuboid'' shape and form domes (*A*). Indirect immunofluorescence studies show that cells possessed large T antigen in all nuclei (*B*), a typical network of cytokeratins (*C*) and tight junctions, evidenced by the ZOI-I labeling of the cells borders and (*D*). Cells grown on filters form confluent monolayers of closely apposed cuboid cells (*E*) separated by tight junctions (arrow) and desmosomes (arrowhead) (*F*). Bars, $A = 50$ μm; *B-D* = 10 μm; *E, F* = 1 μm.

Tag	large T antigen
CFTR	cystic fibrosis transmembrane conductance regulator
db-cAMP	2′-O-dibutyryladenosine 3′-5′-cyclic monophosphate
AVP	arginine vasopressin
dDAVP	deamino-8-D-arginine vasopressin
Am	amiloride
B.Am	benzamyl amiloride
NPPB	5-nitro-2-(3-phenylpropylamino)benzoate
I_{sc}	short-circuit current
R_T	transepithelial electrical resistance
V_T	transepithelial voltage
G_T	transepithelial conductance
ENaC	amiloride-sensitive epithelial Na^+ channel
MR	mineralocorticoid receptor
GR	glucocorticoid receptor.

Results

Morphological and Biochemical Properties of Cultured mpkCCD Cells

The cells proliferatived in vitro and were long-lived (more than 35 passages to date). Cells grown to confluence on Petri dishes had the same shape and formed domes (Fig. 1*A*). Indirect immunofluorescence studies demonstrated that all the nuclei expressed Tag (Fig. 1*B*) and that the cultured cells contained a typical network of cytokeratin (Fig. 1*C*) and ZO-1, a protein associated with the tight junctions (Stevenson et al., 1986), which delineated the cell peripheries (Fig. 1*D*). The mpkCCD cells maintained structural polarity when grown on filters and formed monolayers of closely apposed epithelial cells (Fig. 1*E*) separated by junctional complexes (Fig. 1*F*). Thus, the mpkCCD cells were of epithelial origin and the presence of domes suggested that they had retained their ion transport capacities (Cereijido et al., 1981). The RT-PCR experiments also showed that mpkCCD cells still expressed the α-, β-, and γ-*ENaC* mRNAs (Fig. 2*A*) and the *CFTR* gene (Fig. 2*B*). Table 1 summarizes the cAMP content and the electrophysiological properties of mpkCCD cells. dDAVP, and to a lesser extent isoproterenol, a β-adrenergic agonist acting in CCD (Morel, 1981), increased the cAMP content but not parathormone or calcitonin (Table 1). The mpkCCD cells grown on filters had high R_T and V_T and positive I_{sc} (Table 1).

Effects of Na^+ and Cl^- Channel-Blocking Agents on I_{sc}

The spatial distributions of the ionic conductances in the apical and basolateral membrane domains of mpkCCD cells were defined by measuring I_{sc} in the presence or absence of amiloride (Am) or benzamyl amiloride (B.Am), two ENaC blockers (Kleyman & Cragoe, 1988) or 5-nitro-2-(3-phenylpropylamino)benzoate (NPPB), a potent blocker of Cl^- channels (Wangemann et al., 1986). Am (10^{-6}M) had no effect on I_{sc} when it was applied to the basal side of the cells (Fig. 3). But Am (10^{-6}M) placed on the apical side inhibited I_{sc} by 73% (Fig. 3). Identical results were obtained with B.Am. I_{sc}, as well as the residual amiloride-insensitive component of I_{sc}, was insensitive to 10^{-5}M bumetanide or furosemide, two ''loop'' diuretics inhibitors of the Na^+-K^+-$2Cl^-$ cotransporter, when they were applied to the apical or basal sides of the cells. NPPB (10^{-4}M), added to the apical or basal sides of the cells, inhibited by 26 and 24%, respectively, the I_{sc} measured in basal condition (Fig. 3). Apical addition of 100 μM glibenclamide, a potent inhibitor of ATP-sensitive K^+ channels known to inhibit partially CFTR (Sheppard & Welsh, 1992), slightly reduced I_{sc} by 5%.

Effects of dDAVP on I_{sc}: Modulation by Ion Channel-Blocking Agents and by Substitution of Apical Ions

dDAVP increased I_{sc} in a dose-dependent manner (Fig. 4*A*) as might be expected from its action on cell cAMP

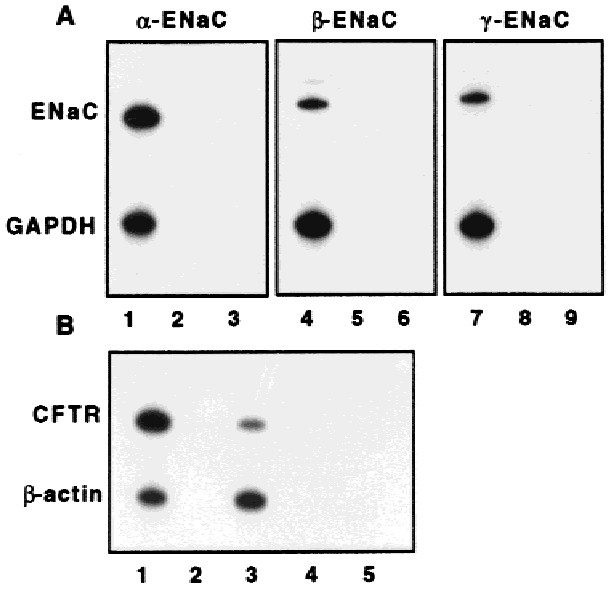

Fig. 2. Expression of *ENaC* and *CFTR* genes in mpkCCD cells. cDNA and nonreverse-transcribed RNA from mpkCCD cells (100 ng) and whole kidney (50 ng) were amplified by RT-PCR. (*A*) Each cDNA sample from mpkCCD cells was amplified (25–28 cycles) with sets of primers specific for α- (lane 1), β- (lane 4), and γ- (lane 7) *rENaC* and *hGAPDH.* Amplified products of expected sizes were obtained with ENaC primers [564-base pair (bp) for α-ENaC, 632-bp for β-ENaC and 647-bp for γ-ENaC]. As control, no amplified product was detected with nonreverse-transcribed RNA (lanes 2, 5, 8) or when cDNA was omitted (lanes 3, 6, 9). B: Each cDNA sample from total kidney (lane 1) and mpkCCD cells (lane 3) was amplified with two sets of primers specific for *CFTR* and β-*actin;* 303-bp amplified products of expected size were obtained with *CFTR* primers (lanes 1 and 3) and no amplified product was detected with nonreverse-transcribed RNA (lanes 2 and 4) or when cDNA was omitted (lane 5). The amplified products from *hGAPDH* and β-*actin* were 320-bp and 250-bp long.

Table 1. cAMP content and transepithelial electrical parameters

cAMP content (pmol/ 7 min/ mg protein)	
Control	22.1 ± 4.1
CT (100 ng/ml)	28.1 ± 7.1
PTH (10^{-7}M)	25.7 ± 5.8
ISO (10^{-6}M)	57.2 ± 12.1[a]
dDAVP (10^{-6}M)	1053.4 ± 86.8[b]
Transepithelial electrical parameters	
R_T (Ω · cm^2)	2798 ± 197
V_T (mV)	−52.0 ± 3.0
I_{sc} (μA/cm^2)	21.5 ± 1.9

The cAMP is that of confluent mpkCCD cells incubated without (Control) or with calcitonin (CT), parathormone (PTH), isoproterenol (ISO) or dDAVP. Values are means ± SE from 6–8 measurements performed on 4–6 different passages. [a]$P < 0.05$, [b]$P < 0.001$ *vs.* control values. R_T, V_T and I_{sc} were measured in basal conditions on confluent cells grown on filters. Values are means ± SE from 42 individual measurements.

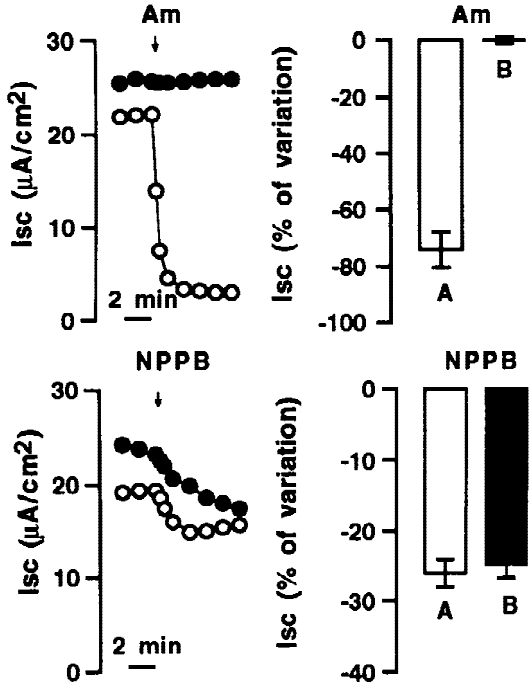

Fig. 3. Effects of ion channel blocking agents on I_{sc}. Representative traces of I_{sc} obtained before and after adding 10^{-6}M amiloride (Am) or 10^{-4}M NPPB to either the apical (○) or basal (●) side of the cells grown on filters. The graphs are the percentage changes in I_{sc} (means ± SE from 6–10 determinations) measured after adding ion channel blocking agents to the apical (*A*) or basal (*B*) solutions.

(Table 1). The half-maximal increase, calculated from the dose-response curve occurred at 8×10^{-10}M dDAVP. Addition (basal and apical) of db-cAMP (5×10^{-3}M) also significantly increased I_{sc} (Fig. 4*B*). These results confirm that dDAVP acts on ionic transport via a cAMP-dependent pathway. All subsequent experiments were therefore performed with 10^{-7}M dDAVP, the concentration which gave maximal rise in I_{sc}. dDAVP added to the basal side caused a rapid rise in I_{sc} (30–60 sec) which was maximal (107 ± 19% above basal I_{sc}, $n = 10$) after 10 min (Fig. 5). B.Am (10^{-6}M) added to the apical side significantly reduced I_{sc} and almost completely prevented the rapid rise induced by dDAVP (Fig. 5). NPPB in the apical bathing solution also blunted I_{sc} and prevented the rise in I_{sc} elicited by dDAVP (Fig. 5), Glibenclamide (100 μM) added to the apical bathing solution, although less efficient than NPPB, also reduced ($p < 0.05$) by 10.2 ± 1.4% ($n = 7$) the rise in I_{sc} induced by dDAVP.

Apical ions replacement experiments were undertaken to determine the contributions of apical Na^+ and Cl^- conductances in the dDAVP-induced changes in I_{sc} (Nagy, Naray-Fejes-Toth, & Fejes-Toth, 1994; Verrey, 1994). Under basal conditions, I_{sc} was significantly decreased (−34%) when Cl^- was replaced by gluconate (control 22.7 ± 2.1; +gluconate: 14.9 ± 1.4 μA/cm^2, $n =$

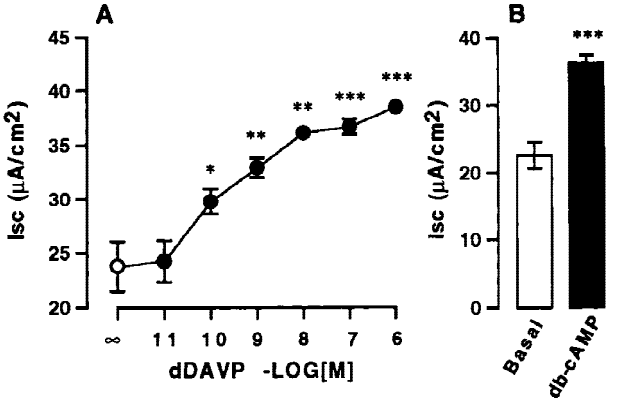

Fig. 4. Effect of dDAVP and db-cAMP on I_{sc}. I_{sc} was measured after adding dDAVP (10^{-11}-10^{-6}M) to the basal side of the cells (*A*). Bars represent the I_{sc} measured in basal condition (Basal) and 10 min after adding 5×10^{-3}M db-cAMP. Values are means ± SE from 6 (*A*) and 4 (*B*) separate experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs.* Basal values.

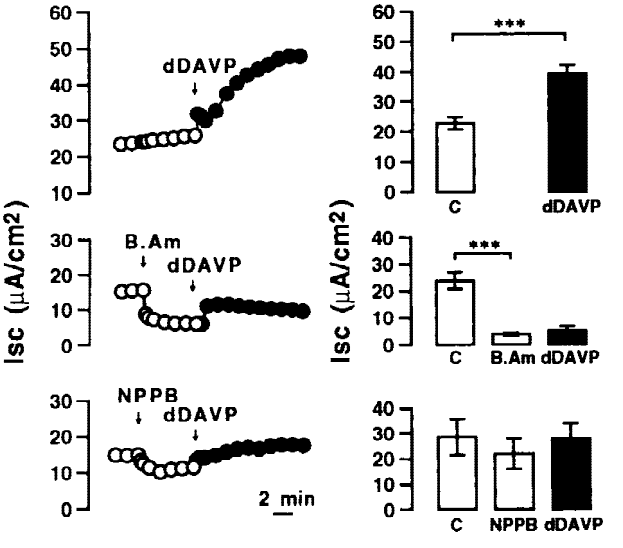

Fig. 5. Influence of ion channel blocking agents on dDAVP-stimulated I_{sc}. Representative traces of I_{sc} measured before (○) and after addition of dDAVP (●) or after adding B.Am (10^{-6}M) or NPPB (10^{-4}M) for 10 min before adding dDAVP. 10^{-7}M dDAVP was always applied to the basal side of the cells, whereas the ion channel blockers were added to the apical side of the cells. Bars represent the mean I_{sc} values ± SE from (5–10) separate experiments. ***$P < 0.001$ *vs.* control (*C*) values.

7, $P < 0.01$). dDAVP still induced a rise in I_{sc} under these conditions (Fig. 6*A*), and subsequent apical addition of B.Am (10^{-6}M) caused a rapid fall in the dDAVP-induced I_{sc} (Fig. 6*A*). Conversely, replacement of apical Na^+ by *N*-methyl-D-glucamine resulted in a dramatic decrease in I_{sc} (−83%) under basal conditions (control: 22.0 ± 3.1; +*N*-methyl-D-glucamine: 3.8 ± 0.3 μA/cm², $n = 6$, $P < 0.001$), and impaired the increase in I_{sc} induced by dDAVP (Fig. 6*B*). Adding NPPB to the apical medium

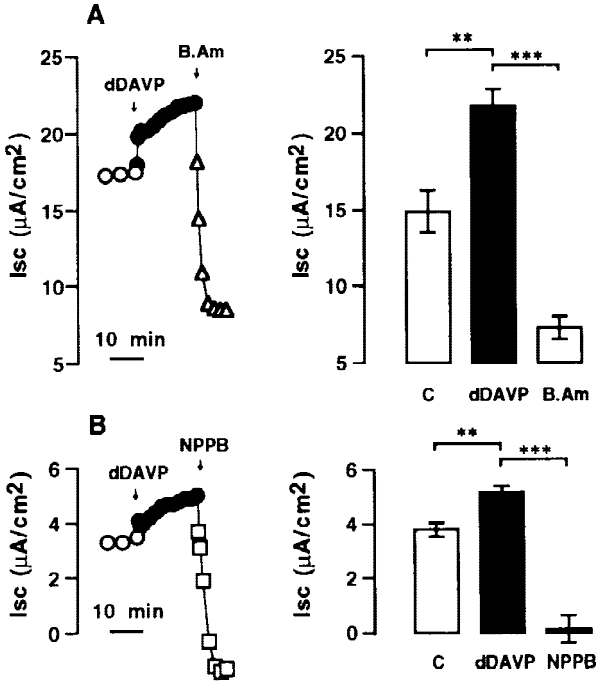

Fig. 6. Effects of apical substitution of Cl^- and Na^+ on I_{sc} elicited by dDAVP. I_{sc} was measured on cells with apical Cl^- replaced by gluconate (*A*) or apical Na^+ replaced by N-methyl-D-glucamine (*B*). *Left panels:* Representative traces of I_{sc} measured before (○) and after the sequential addition of 10^{-7}M dDAVP (●), 10^{-6}M B.Am (△), or 10^{-4}M NPPB (□). dDAVP was applied to the basal side of the cells, and B.Am and NPPB to the apical side of the cells. *Right panels:* Bars represent the mean I_{sc} values ± SE from 5–6 separate experiments. **$P < 0.01$, ***$P < 0.001$ between groups.

fully inhibited the I_{sc} measured after adding dDAVP under the Na^+-free condition, as with B.Am in the Cl^--free condition. Although the I_{sc} values in Cl^-- and Na^+-free conditions were markedly different, I_{sc} was increased by 46% (Cl^--free) and 36% (Na^+-free) after adding dDAVP (Figs. 6*A* and *B*, right panels). These results indirectly suggest that dDAVP increases Na^+ transport and also affects Cl^- fluxes.

Effects of Aldosterone on I_{sc} and $^{22}Na^+$ Transport

As mpkCCD cells have retained features of intact CCD, we examined the capacity of aldosterone to stimulate Na^+ reabsorption. Cells were grown to confluence on filters and then sequentially incubated in steroid-free medium (24 hr) and in hormone-free medium (18 hr). They were then incubated with 5×10^{-7}M aldosterone (Fig. 7). The I_{sc} was slightly lower under basal condition than in cells incubated in HFM alone (*see* Figs. 3 and 4). Aldosterone significantly increased I_{sc} within two hours (Fig. 7*A*); the increase was maximal (3-fold) after 4 hr and remained at a plateau for up to 24 hr. We then next

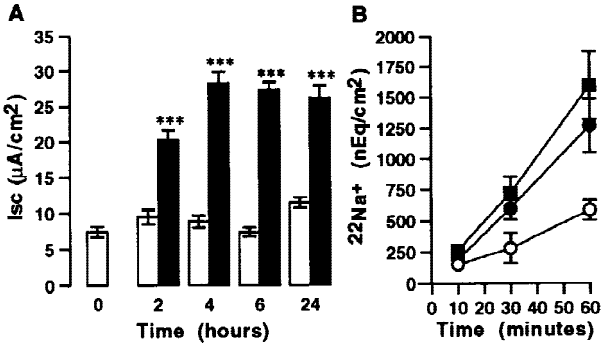

Fig. 7. Effect of aldosterone on I_{sc} and apical-to-basal flux of $^{22}Na^+$. *A:* I_{sc} was measured on confluent cells grown on filters and incubated with (black bars) or without (open bars) 5×10^{-7}M aldosterone for 2 to 24 hr. (*B*) The apical-to-basal flux of $^{22}Na^+$ was measured at intervals on sets of cells incubated without (○) or with 5×10^{-7}M aldosterone for 3 (●) and 24 (■) hr. Values are means ± SE from 6 separate experiments. The amount of $^{22}Na^+$ recovered in the basal medium over 60 min was significantly higher ($P < 0.05$, measured by variance analysis) in the 3 and 24 hr aldosterone-treated cells than in untreated cells.

measured the apical-to-basal flux of $^{22}Na^+$ in untreated cells and in cells incubated with aldosterone for 3 and 24 hr (Fig. 7*B*). The amount of $^{22}Na^+$ recovered in the basal medium increased linearly with time, indicating that the apical-to-basal flux of $^{22}Na^+$ was constant in all conditions tested. The apical-to-basal fluxes of $^{22}Na^+$ measured over 60 min were 2-fold higher in cells incubated with aldosterone for 3 hr than in untreated cells, and 2.7-fold higher in cells incubated with aldosterone for 24 hr. However, the apical-to-basal Na^+ fluxes were not significantly different between cells incubated with aldosterone for 24 and 3 hr. Thus, these results indicate that the mpkCCD cells have retained their amiloride-sensitive Na^+ transport stimulated by aldosterone.

Combined Effects of Aldosterone and Vasopressin on I_{sc}

The experimental protocol used to test the action of aldosterone on I_{sc} was used to analyze the short-term effects of dDAVP on cells incubated with aldosterone for 3 and 24 hr (Fig. 8 and Table 2). dDAVP added to the basal side of the cells treated with aldosterone for 3 hr enhanced I_{sc} by 35%, almost the same extent as in untreated cells (47%). Adding NPPB to the apical side, which reduced the dDAVP-induced I_{sc} by 22% under basal conditions (Table 2), completely inhibited the increase in I_{sc} induced by dDAVP in the 3-hour aldosterone treated cells (Fig. 8). Adding B.Am to the apical medium almost completely inhibited I_{sc} in untreated cells and in those incubated for 3 hr with aldosterone (Fig. 8). Similar results were obtained with cells incubated with aldosterone for 24 hr, although dDAVP increased I_{sc} by only 20%, less than in untreated cells and those incubated with aldosterone for 3 hr (Fig. 8). Adding glibenclamide (100 μM) to the apical medium also reduced by 10% the rise in I_{sc} induced by dDAVP in cells incubated with aldosterone for 3 hr. As in A6 cells (Verrey, 1994), the increase in I_{sc} induced by aldosterone was associated with a significant increase in G_T (Table 2). dDAVP also further increased G_T from untreated and aldosterone-treated cells, whereas B.Am, and to a lesser extent NPPB, reduced it (Table 2). These results indicate that the changes in G_T induced by dDAVP and the ionic channel blockers parallel the changes in I_{sc}. They therefore suggest that, under close-circuit conditions, vasopressin stimulates Cl^-secretion mediated by an apical NPPB- and glibenclamide-sensitive Cl^- conductance in both untreated and aldosterone-treated cells.

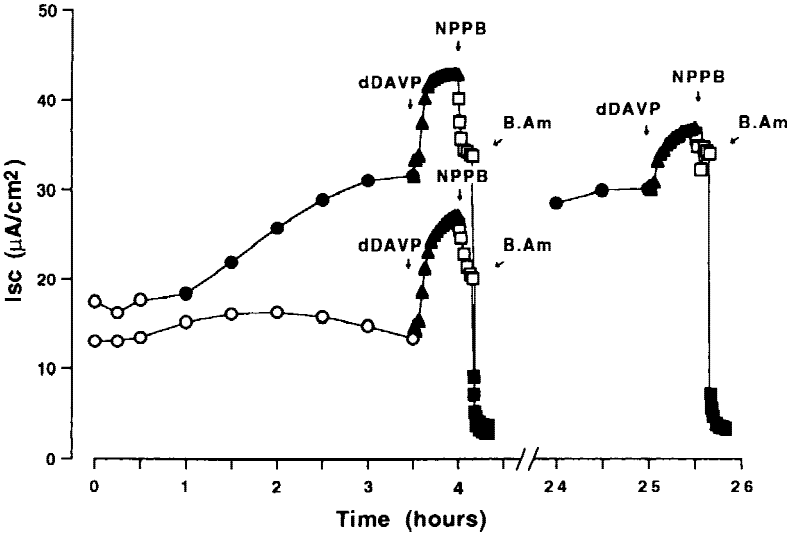

Fig. 8. Effects of dDAVP and aldosterone on I_{sc}. Representative traces of I_{sc} obtained from the cells incubated without (○) or with 5×10^{-7}M aldosterone (●) for 3 hr and 24 hr. After equilibration of the traces, 10^{-7}M dDAVP (▲), 10^{-4}M NPPB (□) and 10^{-6}M B.Am (■) were sequentially added to the basal (dDAVP) or apical sides (NPPB, B.Am) of the cells.

Table 2. Effects of aldosterone and dDAVP on transepithelial electrical parameters

	− Aldosterone		+ Aldosterone (3 hr)		+ Aldosterone (24 hr)	
	I_{sc} (μA/cm^2)	G_T (μS/cm^2)	I_{sc} (μA/cm^2)	G_T (μS/cm^2)	I_{sc} (μA/cm^2)	G_T (μS/cm^2)
None	15.8 ± 1.2	305 ± 18	34.1 ± 3.2	492 ± 35	32.0 ± 1.0	532 ± 16
+ dDAVP	23.3 ± 1.6[b]	433 ± 25[b]	46.2 ± 3.0[a]	607 ± 24[a]	38.4 ± 1.1[b]	597 ± 14[a]
+ NPPB	18.1 ± 1.0[c]	328 ± 29[c]	28.9 ± 2.3[e]	466 ± 27[d]	34.4 ± 1.9	534 ± 26
+ B.Am	2.3 ± 0.3[e]	183 ± 29[e]	2.5 ± 0.2[e]	197 ± 43[e]	3.2 ± 0.1[e]	221 ± 8[e]

Sets of mpkCCD cells were incubated without (−) or with aldosterone (+) for 3 and 24 hr. I_{sc} was measured before (None) and after additions of basal dDAVP (10^{-7}M), apical NPPB (10^{-4}M) and B.Am (10^{-6}M). Values are means ± SE from 6 to 10 separate experiments. [a]$P < 0.05$, [b]$P < 0.01$ *vs.* None values; [c]$P < 0.05$, [d]$P < 0.01$, [e]$P < 0.001$ *vs.* dDAVP values.

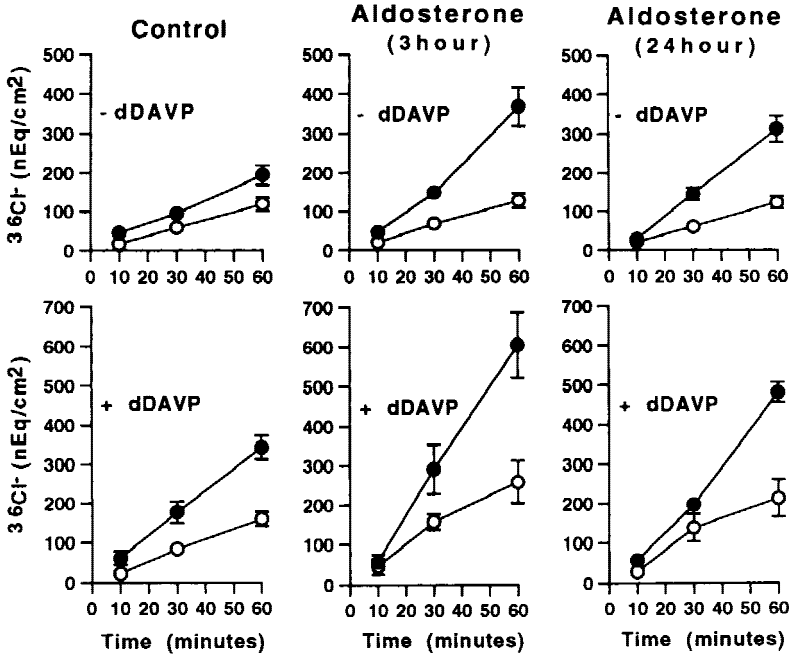

Fig. 9. Effects of dDAVP and aldosterone on bidirectional transport of $^{36}Cl^-$. Basal-to-apical (○) and apical-to-basal (●) transport of $^{36}Cl^-$ were measured from 10 to 60 min on cells incubated without (Control) or with 5×10^{-7} M aldosterone for 3 and 24 hr in the absence (− dDAVP) or presence (+ dDAVP) of 10^{-7}M dDAVP added to the basal side of the cells. Values are means ± SE from 4 to 6 experiments.

Effects of Aldosterone and Vasopressin on Chloride Fluxes

The bidirectional transport of $^{36}Cl^-$ in untreated and aldosterone-treated cells were measured to test the effects of aldosterone and dDAVP on ion transport under more physiological conditions (Fig. 9 and Table 3). The apical-to-basal and basal-to-apical transport of $^{36}Cl^-$ increased linearly over 60 min (Fig. 9). The apical-to-basal transport of $^{36}Cl^-$ was slightly higher than the basal-to-apical transport of $^{36}Cl^-$ under basal conditions (Table 3). The apical-to-basal Cl^- flux was greater ($P < 0.05$) in cells treated with aldosterone for 3 or 24 hr than in untreated cells, whereas the basal-to-apical flux of $^{36}Cl^-$ remained unchanged (Table 3). As a consequence, the apical-to-basal over basal-to-apical Cl^- flux ratio was about 1.7-fold greater in aldosterone-treated than in untreated cells (Table 3). Under basal conditions, dDAVP added to the basal medium increased both bidirectional transport of $^{36}Cl^-$ with a preferential and significant increase in the apical-to-basal transport of $^{36}Cl^-$ (Fig. 9 and Table 3). dDAVP also increased both apical-to-basal and basal-to-apical transport of $^{36}Cl^-$ in cells incubated with aldosterone for 3 and 24 hr (Fig. 9 and Table 3). As in untreated cells, the increase in the apical-to-basal transport of Cl^- in aldosterone-treated cells induced by dDAVP was much higher than the basal-to-apical transport of Cl^-. As a result, the apical-to-basal over basal-to-apical Cl^- flux ratio measured after adding dDAVP to the basal side in untreated and aldosterone-treated cells were almost identical (Table 3). In all cases, the apical-to-basal flux of $^{36}Cl^-$ was always higher than the basal-to-apical flux, resulting in net Cl^- absorption. Figure 10 summarizes the net $^{36}Cl^-$ reabsorption measured in the

Table 3. Effects of aldosterone and dDAVP on the unidirectional fluxes of $^{36}Cl^-$

	Apical-to-Basal	Basal-to-Apical	A-B/B-A
	$^{36}Cl^-$ flux (nEq. 60 $min^{-1} \cdot cm^{2-1}$		
Control	194.9 ± 24.9	119.1 ± 17.2	1.6
+ dDAVP	343.3 ± 30.2[a]	160.4 ± 18.1[b]	2.1
Aldosterone (3 hr)	369.4 ± 49.6	128.3 ± 10.1[b]	2.9
+ dDAVP	604.3 ± 82.1[a]	259.3 ± 53.8[b]	2.4
Aldosterone (24 hr)	313.7 ± 33.6	123.9 ± 16.6[b]	2.5
+ dDAVP	481.3 ± 25.3[a]	212.8 ± 47.8[b]	2.3

Apical-to-basal and basal-to-apical $^{36}Cl^-$ fluxes were measured on sets of confluent cells grown on filters over a period of 60 min under basal condition (Control) and in cells incubated with 5×10^{-7}M aldosterone for 3 and 24 hr. In each condition tested, $^{36}Cl^-$ fluxes were also measured after basal addition of 10^{-7}M dDAVP. Results are means ± SE from 5–6 experiments. A-B over B-A represents the apical-to-basal over basal-to-apical $^{36}Cl^-$ flux ratio. [a] and [b] indicate significant differences ($P < 0.05$, measured by variance analysis) between I_{sc} values from untreated and dDAVP-treated cells ([a]) or apical-to-basal and basal-to-apical $^{36}Cl^-$ fluxes ([b]).

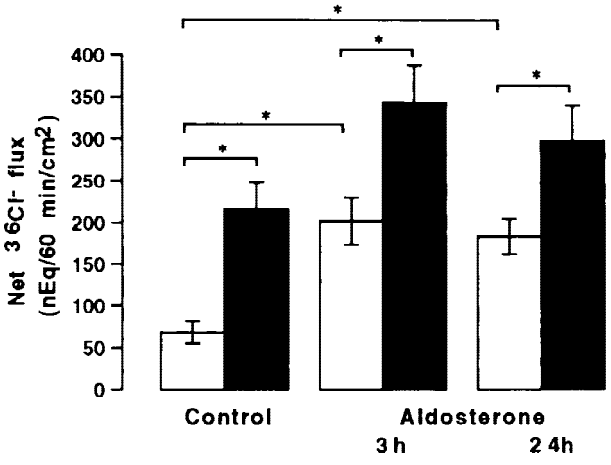

Fig. 10. Effects of dDAVP on net fluxes of $^{36}Cl^-$ in untreated and aldosterone-treated cells. The net flux of $^{36}Cl^-$ (expressed as nEq per cm^2 surface filter per 60 min) was calculated from the differences between apical-to-basal and basal-to-apical $^{36}Cl^-$ fluxes measured in the absence (open bars) or presence (black bars) of 10^{-7}M dDAVP in untreated (Control) and aldosterone-treated cells for 3 and 24 hr. In both conditions, cells exhibited net Cl^- reabsorption which increased after addition of dDAVP. Values are means ± SE from 4 experiments. * Represents statistical significance ($P < 0.05$, measured by variance analysis) between groups.

absence or presence of dDAVP on other sets of cells treated with aldosterone or untreated cells. Cl^- reabsorption increased significantly in the 3 hr (202.4 ± 27.9 nEq. 60 $min^{-1} \cdot cm^{2-1}$, $n = 4$) and 24 hr (183.7 ± 21.4 nEq. 60 $min^{-1} \cdot cm^{2-1}$, $n = 4$) aldosterone-treated cells as compared to untreated cells (68.2 ± 13.1 nEq. 60 $min^{-1} \cdot cm^{2-1}$, $n = 4$). dDAVP, which significantly increased net Cl^- flux in the absence of aldosterone, further increased to almost the same extent the net Cl^- flux in both untreated and aldosterone-treated cells (Fig. 10).

Discussion

The particular feature of the SV-PK/Tag transgene to be expressed in the distal segments of the nephron from transgenic mice fed a carbohydrate-rich diet (Miquerol et al., 1996) was used to establish a line of immortalized renal epithelial cells that retained the morphological and functional properties of the native CCD from which they were derived. The mpkCCD cells have the typical properties of a corticosteroid-sensitive tight epithelium and contain the three α-, β- and γ-*ENaC* mRNAs subunits, normally located in the apical membrane of CCD (Duc et al., 1994). The mpkCCD cells also express the *CFTR* gene as do primary cultures of rabbit and immortalized mouse M-1 CCD cells (Todd-Turla et al., 1996).

Aldosterone (5×10^{-7}M) caused a rapid (less than 2 hr) 3-fold increase in I_{sc} and apical-to-basal flux of $^{22}Na^+$. Aldosterone acts on sodium transport by initially binding to corticosteroid receptors (Rossier & Palmer, 1992; Verrey, 1995). Kidney CCD, like other target tissues for corticosteroid hormones, possess two types of corticosteroid receptors: high affinity low-capacity Type I receptors (MR) and low affinity high-capacity Type II receptors (GR) (Kusch, Farman & Edelman, 1978; Farman, Vandewalle & Bonvalet, 1982; Claire et al., 1989). It is generally agreed that aldosterone acts by occupying Type I receptors (MR), but several studies on A6 cells (Watlington et al., 1982; Duncan et al., 1988; Schmidt et al., 1993) and primary cultures of CCD (Naray-Fefes-Toth & Fejes-Toth, 1990) have shown that glucocorticosteroid hormones may stimulate Na^+ transport by binding to GR. The concentration of aldosterone required to elicit a significant rise in I_{sc} was relatively high (5×10^{-7}M) in this study, but close to that required (3×10^{-7}M) to elicit maximal sodium transport in A6 cells (Verrey et al., 1987). The differences in the affinities of aldosterone for the Type I (0.1–1 nM) and Type II (5–10 nM) receptors measured by binding and competition studies on A6 and rabbit CCD (Farman, Vandewalle & Bonvalet, 1982; Claire et al., 1989), suggest that the increase in sodium transport induced by aldosterone in mpkCCD cells is presumably mediated by the binding of aldosterone to GR.

Several studies have demonstrated that AVP or vasotocin induces a twofold increase in Na^+ channel activity within 5 to 20 min, which corresponds to an increase in apical Na^+ channel density (for review *see* Garty & Palmer, 1997). Vasopressin acts in synergy with corticosteroid hormones to increase sodium transport in vivo (Reif & Schafer, 1986). dDAVP also produces a rapid

increase in I_{sc} in vitro and potentiates the action of aldosterone. Similar results have been reported for primary cultures of CCD cells treated with aldosterone for 48 hr (Canessa & Schafer, 1992) and for A6 cells treated for five days with aldosterone (Verrey, 1994). The rise in I_{sc} induced by dDAVP in our study is partially inhibited by NPPB and almost completely abolished by B.Am. Thus, there is a Cl^- secretion induced by vasopressin when the electrochemical gradient favors Cl^- secretion under close-circuit conditions (Husted et al., 1995). Vasopressin also modifies the Cl^- flux from untreated and aldosterone-treated cells. Kizer et al. (1995) reported that aldosterone increased sodium reabsorption in immortalized mouse IMCD cells and that these cells may secrete Cl^- by an electrogenic mechanism. Verrey (1994) reported different effects of vasotocin on I_{sc}, performed on A6 cells in close-circuit conditions, and on bidirectional Cl^- flux experiments performed in the more physiological open-circuit conditions. The driving forces depending on the activation of Na^+ channels which depolarize the apical membrane, favor Cl^- reabsorption in physiological conditions. Measurements of unidirectional $^{36}Cl^-$ fluxes under open-circuit conditions show that mpkCCD cells have a modest but predominant apical-to-basal flux of Cl^- under basal conditions, and that dDAVP mostly increases this flux. Aldosterone *per se* also significantly enhances the apical-to-basal flux of Cl^- without affecting Cl^- flux in the opposite direction.

Exactly how vasopressin potentiates the action of aldosterone on sodium transport is still not clear. Chronic administration of deoxycorticosterone seems to potentiate the increase in cAMP in response to vasopressin (McArdle et al., 1992). But, as indicated by Hawk et al. (1996), this appears to be unlikely, since we found no differences in the amounts of cAMP with various concentrations of dDAVP (10^{-10}–10^{-6}M) in untreated cells or in aldosterone-treated cells (*data not shown*). The Cl^- channel(s) mediating the increase in Cl^- transport elicited by vasopressin and aldosterone has not yet been identified. Several Cl^- conductances have been characterized in intact and primary cultures of CCD cells. A 46 pS chloride channel has been identified in the basolateral membrane of rabbit CCD principal cells (Sansom, La & Carosi, 1990). Ling et al. (1994) have described a 9 pS Cl^- channel in the apical membrane of primary cultured rabbit CCD cells, that is activated by PGE2, forskolin and cAMP. Intercalated cells have an apical membrane Cl^- channel modulated by a pertussis toxin sensitive G-protein and activated by adenosine (Schwiebert et al., 1990). Two Cl^- channels belonging to the C1C chloride channel family, rC1C-K1 and rC1C-K2 channel proteins, are also present in the basolateral membranes of rat CCD (Vandewalle et al., 1997), but their function has not been demonstrated unambiguously (Kieferle et al., 1994; Uchida et al., 1995). CFTR, believed to act as a cAMP-regulated Cl^- conductance (Bradbury et al., 1992), is present in intact and cultured CCD, mainly in β-intercalated cells (Todd-Turla et al., 1996). The physiological role of CFTR in the kidney is still unclear, but it has been recently shown that a cAMP-dependent reciprocal regulation of ENaC and CFTR Cl^- channels may occur in apical membrane of respiratory and CCD epithelial cells (Stutts et al., 1995; Letz & Korbmacher, 1997). A Cl^- channel with features very similar to those of the CFTR has been identified in the apical membranes of immortalized mouse CCD and IMCD cell lines (Husted et al., 1995; Letz & Korbmacher, 1997). The fact that NPPB and to a lesser extent glibenclamide, which partially inhibits the CFTR (Sheppard & Welsh, 1992), reduce the rise in I_{sc} induced by vasopressin suggests that the fractional increase in basal-to-apical Cl^- flux induced by vasopressin is mediated in part by CFTR (Husted et al., 1995; Letz & Korbmacher, 1997). In contrast, aldosterone increases the apical-to-basal Cl^- flux, but does not alter the basal-to-apical component of the Cl^- flux. This suggests that apical Cl^- conductances, including CFTR, are not affected by the steroid hormone. We also find that the amounts of *CFTR* transcripts in untreated and aldosterone-treated cells are identical (*data not shown*). Results from net Cl^- flux experiments clearly show that dDAVP further enhances the increase in Cl^- reabsorption induced by aldosterone. Hence, this observation may raise the question of whether aldosterone activates the transcription of a specific class of Cl^- channels expressed in CCD cells.

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