Adenosine Regulates a Chloride Channel via Protein Kinase C and a G Protein in a Rabbit Cortical Collecting Duct Cell Line

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Abstract

We examined the regulation by adenosine of a 305-pS chloride (Cl^-) channel in the apical membrane of a continuous cell line derived from rabbit cortical collecting duct (RCCT-28A) using the patch clamp technique. Stimulation of A_1 adenosine receptors by N^6-cyclohexyladenosine (CHA) activated the channel in cell-attached patches. Phorbol 12,13-didecanoate and 1-oleoyl 2-acetylglycerol, activators of protein kinase C (PKC), mimicked the effect of CHA, whereas the PKC inhibitor H7 blocked the action of CHA. Stimulation of A_1 adenosine receptors also increased the production of diacylglycerol, an activator of PKC. Exogenous PKC added to the cytoplasmic face of inside-out patches also stimulated the Cl^- channel. Alkaline phosphatase reversed PKC activation. These results show that stimulation of A_1 adenosine receptors activates a 305-pS Cl^- channel in the apical membrane by a phosphorylation-dependent pathway involving PKC. In previous studies, we showed that the protein $G_{\alpha i\text{-}3}$ activated the 305-pS Cl^- channel (Schwiebert et al. 1990. *J. Biol. Chem.* 265:7725–7728). We, therefore, tested the hypothesis that PKC activates the channel by a G protein–dependent pathway. In inside-out patches, pertussis toxin blocked PKC activation of the channel. In contrast, H7 did not prevent G protein activation of the channel. We conclude that adenosine activates a 305-pS Cl^- channel in the apical membrane of RCCT-28A cells by a membrane-delimited pathway involving an A_1 adenosine receptor, phospholipase C, diacylglycerol, PKC, and a G protein. Because we have shown, in previous studies, that this Cl^- channel participates in the regulatory volume decrease subsequent to cell swelling, adenosine release during ischemic cell swelling may activate the Cl^- channel and restore cell volume. (*J. Clin. Invest.* 1992. 89:834–841.) Key words: cell culture • intercalated cells • ion channels • RCCT-28A cells • regulatory volume decrease • signal transduction

Introduction

Adenosine is produced by all cells and has numerous physiological actions including vasodilatation and vasoconstriction, inhibition of neurotransmission, platelet aggregation, lipolysis, and stimulation of glucose oxidation (1). The renal actions of adenosine include vasoconstriction or vasodilatation, redistribution of renal blood flow, inhibition of renin release, inhibition of neurotransmitter release from renal nerves, and changes in solute and water excretion (1). Elucidation of the renal tubular effects of adenosine is complicated by hormonally induced changes in hemodynamics and glomerular filtration rate that can mask direct effects of adenosine. Nevertheless, adenosine has been shown to antagonize vasopressin-stimulated water reabsorption in the cortical collecting duct (CCD)[1] (2) and inner medullary collecting duct (3), to stimulate Na^+-phosphate and Na^+-glucose transport in opossum kidney cells (4), and to inhibit the transepithelial voltage across isolated cortical thick ascending limbs of Henle's loop (5), suggesting a direct inhibitory action on sodium chloride reabsorption.

Adenosine is formed within renal cells and enters the extracellular space by facilitated diffusion via a nucleoside transporter (1). Production and release of adenosine is increased by anoxia (1). In the extracellular space, adenosine activates specific membrane receptors (A_1 and A_2). It is inactivated mainly by cell uptake via the nucleoside transporter (1). Stimulation of A_1 receptors antagonizes adenylyl cyclase and decreases cAMP generation, whereas stimulation of A_2 receptors enhances adenylyl cyclase activity and increases cAMP production (1). Activation of A_1 receptors also enhances the turnover of inositol phosphates and increases cell calcium (1, 6–8). Although the functional significance of A_1 and A_2 receptors in the CCD is not entirely clear (1, 6–8), the ability of adenosine to regulate basal and hormone-stimulated cAMP production via activation of its two receptors and its ability to stimulate calcium and inositol phosphate turnover make it a potentially important modulator of hormonally regulated solute and water transport in this nephron segment (1).

In previous studies, we demonstrated that a large-conductance Cl^- channel (305 pS) in the apical cell membrane of RCCT-28A cells, a continuous cell line derived from rabbit CCD, is normally inactive in the basal state. The Cl^- channel is activated by cell swelling, membrane stretch, and the GTP-binding protein, $G_{\alpha i\text{-}3}$ (9–15). Because conductive Cl^- secretion is regulated by adenosine in a variety of epithelia (16–18), the present study was conducted to determine whether adenosine

Preliminary data of this work have been published in abstract form (1990. *J. Am. Soc. Nephrol.* 1:481; 1990. *J. Am. Soc. Nephrol.* 1:692; 1990. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:830).

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Received for publication 13 August 1991 and in revised form 5 November 1991.

J. Clin. Invest.

0021-9738/92/03/0834/08 $2.00
Volume 89, March 1992, 834–841

1. *Abbreviations used in this paper:* CCD, cortical collecting duct; CHA, N^6-cyclohexyladenosine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; DAG, *sn*-1,2-diacylglycerol; DDA, 2',3'-dideoxyadenosine; DiC8, 1,2-dioctanoylglycerol; DNSA, 1-dimethylamino-naphthalene-5-sulfonamide; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; H8, *N*[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; IC, intercalated cells (similarly, A-IC and B-IC, acid-secreting and base-secreting IC); OAG, 1-oleoyl 2-acetyl glycerol; PDD, phorbol 12,13-didecanoate; PKC, protein kinase C; PTX, pertussis toxin.

regulates Cl^- channels in RCCT-28A cells and to examine the signaling mechanisms involved in this regulation. We report that adenosine activates the 305-pS channel by a membrane-delimited pathway involving A_1 receptor, phospholipase C, diacylglycerol (DAG), protein kinase C (PKC), and $G_{\alpha i\text{-}3}$.

Methods

Cell culture. As described previously, CCD cells were immunodissected from rabbit kidney and infected with an adenovirus 12-simian virus 40 hybrid, resulting in a continuous cell line designated RCCT-28A (6). RCCT-28A cells were grown in Dulbecco's modified Eagle's medium (DME; Hazelton Biologics, Inc., Lenexa, KS) supplemented with $NaHCO_3$ (24 mM; pH 7.4 gassed with 5% CO_2/air at 37°C), 10% heat-inactivated fetal bovine serum (Hyclone Laboratories Inc., Logan, UT), L-glutamine (2 mM), dexamethasone (1 μM), penicillin-G (50 U/ml), and streptomycin (50 U/ml). The cells were grown on glass coverslips coated with plating medium (Vitrogen 100 [1 ml/102 ml DME; Collagen Corporation, Palo Alto, CA], bovine serum albumin (BSA; 10 μg/ml), human fibronectin [10 mg/ml; Collaborative Research, Bedford, MA]) or on uncoated, permeable Falcon Cyclopore cell culture inserts (Becton Dickinson Labware, Lincoln Park, NJ) and studied between passages 7 and 25.

Characterization of RCCT-28A cells. In a previous study, Arend et al. (6) showed that RCCT-28A cells retain their epithelial morphology, are recognized by a monoclonal antibody specific for the CCD, IgG_2 (rct-30A), but not by monoclonal antibodies specific for proximal tubule, thick ascending limb, or mesangial cells. Furthermore, RCCT-28A cells have A_1 and A_2 adenosine receptors, bind peanut lectin agglutinin, a selective marker of intercalated cells (IC) but not principal cells in rabbit CCD (6, 19–21) and they secrete H^+ by an electrogenic mechanism (22, 23). These observations suggest that RCCT-28A cells are phenotypically most similar to acid-secreting IC (A-IC) in the CCD. In the present study we extend the initial characterization of RCCT-28A cells by using a fluorescent marker for carbonic anhydrase and a panel of monoclonal antibodies.

Determination of carbonic anhydrase activity in RCCT-28A cells. To determine if the cells contain carbonic anhydrase, an enzyme present in IC but not in rabbit principal cells (24, 25), we stained cells with 1-dimethylamino-naphthalene-5-sulfonamide (DNSA), a fluorescent analogue of acetazolamide, which binds to carbonic anhydrase (26). Cells were washed in phosphate-buffered saline (PBS; 4°C), fixed in paraformaldehyde (1%) in PBS (4°C) for 30 min, and stained for 2 h with DNSA (10^{-5} M). To determine whether DNSA binding was specific, some cells were preincubated with acetazolamide (10^{-3} M) at 4°C and subsequently washed twice with PBS (4°C) before incubation with DNSA. Epifluorescence was examined using a Cytofluorograph System 50H flow cytometer interfaced with a model 2150 computer (Ortho Diagnostic Systems, Westwood, MA). The filters used to visualize DNSA were a 360-nm band pass filter for excitation, a 395-nm dichroic mirror, and a 470 band-pass filter for emission. More than 95% of cells specifically bound DNSA.

Immunocytochemistry. Cells were grown on either glass coverslips coated with Vitrogen plating medium or Cyclopore cell culture inserts and fixed by the periodate-lysine-paraformaldehyde method for 1 h (25). This fixative is composed of 0.01 M $NaIO_4$, 0.75 M lysine, and 2% paraformaldehyde in 0.375 M phosphate buffer (pH 7.4). After fixation, cells were washed three times in PBS and subsequently permeabilized with 0.1% Triton-X100 in PBS (5 min). Cells were rinsed three times with PBS/1% BSA and incubated with the primary antibody for 30 min at room temperature. After three washes in PBS/BSA, the cells were incubated for 30 min with a 1:100 dilution of a FITC-labeled goat anti-mouse IgG antibody and then rinsed again in PBS. Cells were placed in a Tris (200 mM)/50% glycerol solution (pH 8.0) containing 1% *n*-propylgallate to retard fading and examined with a microscope (Diaphot; Nikon, Inc., Melville, NY) with epiflourescence (×400 and ×1,000) and Hoffman modulation contrast optics.

We used several cell-specific monoclonal antibodies to characterize RCCT-28A cells: IVF12 (generous gift of Dr. M. Jennings [19]), an antibody directed against the membrane domain of human erythrocyte band 3 protein that was used as a marker of A-IC in rabbit CCD (19); F13-631 and F13-601, which specifically label base-secreting IC (B-IC) in rabbit CCD (21); F13-1201, F3-101, F6-381, and F6-1242, which specifically label principal cells in rabbit CCD (21); and mr-mct, which specifically labels A-IC (27). The antibodies of the F series were generous gifts of Dr. Geza Fejes-Toth, Dartmouth Medical School. Experimental results were similar for cells grown on coated-glass coverslips and Cyclopore filters.

All cells were recognized by IVF12 (19).[2] In contrast, the four antibodies specific for rabbit principal cells did not recognize RCCT-28A cells. Furthermore, antibodies specific for B-IC did not label the cells. However, mr-mct, a marker for A-IC (27), labeled all cells. These observations confirm that RCCT-28A cells are phenotypically similar to A-IC in the CCD.

Analysis of Cl^- channels. Single-channel currents in the apical membrane of RCCT-28A cells were measured with a current-to-voltage converter (model PC-501, Warner Instrument Corp., Hamden, CT), low pass-filtered at 300 Hz, and digitized at 1 kHz with an IBM AT computer (PClamp Version 5.5, Axon Instruments, Burlingame, CA) as described in detail previously (9, 10). Briefly, the single-channel current amplitude was calculated by constructing amplitude histograms of single-channel currents (i). Channels were considered open when the current was larger than $i/2$. Data were recorded for a minimum of one 10-s trial every minute during control and experimental periods. Single-channel open probability (P_o) was defined as the total time the channel was open divided by the total time of data collection. In patches containing multiple channels (~ 8% of all patches), the P_o was calculated as described (28). During control periods (> 2–5 min), the P_o remained constant in each membrane patch. Single-channel currents were also displayed continuously on a strip chart recorder throughout the control and experimental period (> 5–10 min). Currents were recorded at a command voltage of −20 or −30 mV (cytoplasmic side of the membrane patch negative relative to the interior of the pipette); this voltage is similar to the membrane potential measured in IC in isolated CCD (29). Data on P_o are presented as mean±SE during the control and experimental periods. The statistical significance of an experimental procedure was determined using paired Student's t test; a P value < 0.05 was considered significant.

The patch pipettes were filled with (in mM) NaCl 140, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, Hepes 10, titrated to pH 7.4 with NaOH. The same solution was present in the bath during gigaohm seal formation. When membrane patches were excised to form the inside-out configuration, the bath solution containing (in mM) NaCl 5, KCl 140, $CaCl_2$ 1, $MgCl_2$ 1, Hepes 10, titrated to pH 7.4 with KOH. In some experiments, the bath solution calcium activity was adjusted to 100 or 200 nM by adding calculated amounts of calcium to solutions containing 2 mM EGTA (28). Experiments were conducted in a paired fashion at 23°C.

Analysis of DAG production. The effect of the adenosine analogue N^6-cyclohexyladenosine (CHA) on DAG accumulation was determined as reported by Griendling et al. (30). RCCT-28A cells were grown to ≈ 90% confluence on 100-mm petri dishes in supplemented DME medium described above, and labeled with [^{3}H]arachidonic acid (0.5 μCi/ml [New England Nuclear, Boston, MA]) for 4 h before addition of agonist. After washing, cells were exposed to fresh medium containing CHA for times and at concentrations indicated in Results. The experiment was terminated by addition of ice-cold H_2O (2.5 ml). Subsequently, cells were scraped and mixed via trituration and an aliquot was removed for protein determination. The remaining sample

2. In a previous study (6), IVF12 did not recognize RCCT-28A cells; however, in that study, in contrast to the present work, the cells were not permeabilized with Triton-X100 before application of the primary antibody. IVF12 was localized to the basolateral membrane region of RCCT-28A cells.

was placed in a glass centrifuge tube and extracted with chloroform/methanol/H_2O (6.5:5:2). The $CHCl_3$ was evaporated under N_2 and the sample was resuspended in 75 μl of $CHCl_3$ and separated by thin-layer chromatography on channeled, silica gel G plates (Analtech, Inc., Newark, DE). The mobile phase consisted of benzene/diethylether/ammonia 100:80:0.2. The spots were visualized with iodine vapors and those corresponding to standards were scraped and quantified by liquid scintillation spectrophotometry.

Materials. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, unless otherwise noted. The A protomer of pertussis toxin was obtained from List Biological Laboratories, Inc., Campbell, CA. The α_{i-3} subunit of G_i was a generous gift from Dr. Lutz Birnbaumer and his colleagues, and was purified from human erythrocytes and activated with GTPγS (31). 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7) and *N*-[2-(methylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride (H8) were obtained from Seikagaku America, Inc., St. Petersburg, FL. Exogenous purified isoforms of PKC were obtained from Lipidex, Inc., Westfield, NJ (multiple isozymes of PKC purified from brain; specific activity = 2,560 U/mg). PKC and α_{i-3} were added to a static bath solution, which corresponds to the cytoplasmic face of inside-out patches.

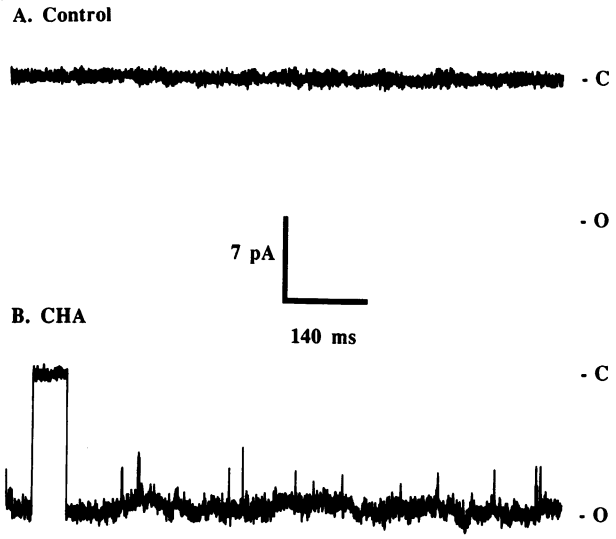

Figure 1. Representative single-channel currents records illustrating the effect of CHA on the 305-pS Cl^- channel in the apical membrane of cell-attached patches. (*A*) Control, no channel activity. (*B*) 2 min after the addition of CHA (10^{-6} M) to the bath solution, the P_o increased dramatically. "O" indicates the open state and "C" indicates the closed state. The membrane was hyperpolarized by 50 mV from the resting membrane potential.

Results

Adenosine effects on the 305-pS Cl^- channel. As described previously, we found that the 305-pS Cl^- channel in the apical membrane was rarely active in unstimulated cells (9, 10). The first series of experiments were conducted to determine whether adenosine activates the Cl^- channel. The addition of the poorly hydrolyzable A_1 adenosine receptor agonist CHA (10^{-7} or 10^{-6} M) to the bath solution activated quiescent Cl^- channels in cell-attached patches (Fig. 1). The P_o of the channel increased from 0 to 0.42 (Fig. 2 *A*). To determine if the effect of CHA was specific for A_1 receptors, we pretreated cells with the selective A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (CPX; 2×10^{-7} M) and then added CHA. CPX alone (15 min) had no effect on the P_o but completely blocked the action of CHA (Fig. 2 *B*). These results indicate that CHA activates the Cl^- channel via stimulation of A_1 receptors.

It is not known whether A_1 receptors are located on the apical and/or basolateral membrane in RCCT-28A cells. We therefore conducted experiments to determine whether they are located in the apical membrane. In unpaired experiments, we added CHA (10^{-6} M) to the solution in the patch electrodes before forming a gigaohm seal. In the absence of CHA in the pipette and bath solution, the 305-pS Cl^- channel was rarely active in the cell-attached configuration (n = 3/21 patches containing Cl^- channels). In contrast, with CHA in the pipette, significantly more chloride channels were active in cell-attached patches (n = 8/18 patches containing Cl^- channels; $P < 0.001$ by χ^2 analysis). Because these experiments were conducted in a continuously flowing bath solution (2–5 ml/min), it is unlikely that potential leakage of CHA from the patch pipette into the bath solution resulted in CHA concentrations sufficient to stimulate adenosine receptors in the basolateral membrane. These results suggest that A_1 receptors are located on the apical membrane; however, we cannot exclude the possibility that they are also present in the basolateral membrane.

Stimulation of A_1 adenosine receptors may activate the Cl^- channel by increasing phospholipase C activity or by inhibiting adenylyl cyclase (1, 6). To discriminate between these possible actions of A_1 receptor stimulation, we added the adenylyl cyclase inhibitor, 2',3'-dideoxyadenosine (DDA; 10^{-4} M) (32) to the bath solution of cell-attached patches. DDA did not increase the P_o of quiescent Cl^- channels (n = 5; P_o was 0.00 in control and 0.01±0.01 after 15 min of DDA exposure).[3] Furthermore, pretreatment with DDA for 15 min did not alter the stimulation by CHA of the P_o (n = 3). These observations suggest that CHA increases the P_o by binding to A_1 adenosine receptors and activating phospholipase C.

Activation of phospholipase C increases the cellular production of inositol phosphates and diacylglycerol, a second messenger that stimulates PKC (1). To determine whether CHA activated the Cl^- channel by a pathway involving phospholipase C and PKC, we added the protein kinase inhibitor H7 (25 μM) to the bath solution of cell-attached patches 15 min before adding CHA. As illustrated in Fig. 2 *C*, H7 attenuated CHA activation of the channel, suggesting that CHA increases the P_o by activating a protein kinase. Support for PKC activation of the channel is provided by experiments with phorbol 12,13-didecanoate (PDD, 10 nM) and 1-oleoyl 2-acetyl glycerol (OAG, 0.1 mM). As illustrated in Fig. 3, both activators of PKC increased the P_o in cell-attached patches. The protein kinase inhibitor H8 (25 μM) reversed PDD activation (P_o was 0.06±0.05 in control, 0.26±0.09 with PDD and 0.05±0.03 with H8; $P < 0.05$; n = 3). The inactive phorbol ester, 4α-phorbol 12-myristate 13-acetate (αPMA; 10 nM), had no effect on the P_o in cell-attached patches (P_o was 0.16±0.04 in control and 0.15±0.05 with αPMA; n = 3). These experiments demonstrate that PKC activates the Cl^- channel.

To provide additional support for the view that PKC activates the Cl^- channel, we added exogenous PKC (isolated and purified from bovine brain; 0.8 U/ml) to the cytoplasmic face of excised, inside-out patches. PKC, in the presence of ATP (1

3. The presence of a channel in the membrane was confirmed by subsequent excision and activation by a depolarizing voltage step.

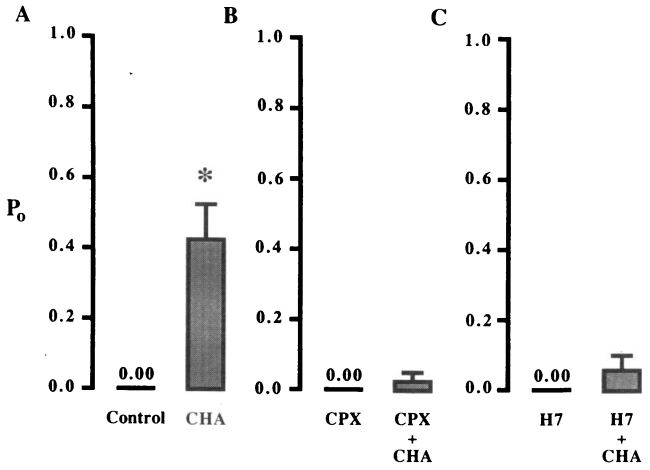

Figure 2. A_1 receptor agonist (CHA) specifically activates the 305-pS Cl^- channel. (*A*) CHA (10^{-7} or 10^{-6} M) increased the P_o within 2–3 min from 0.00 to 0.42±0.01 ($P < 0.01$; $n = 10$). (*B*) CPX (2×10^{-7} M), a specific A_1 receptor antagonist, had no effect on the P_o (0.00 in control and 0.00 with CPX; $n = 5$); however, CPX prevented the CHA-induced increase in the P_o (0.02±0.02 with CPX and CHA; $n = 5$). (*C*) H7 (25 μM), an inhibitor of protein kinases, prevented the CHA-induced increase in the P_o. The P_o was 0.00 in control, 0.00 after H7 treatment, and 0.06±0.04 after H7 and CHA ($n = 5$). The membrane voltage was hyperpolarized in cell-attached patches by 40 mV from the resting membrane potential.

mM) and 1,2-dioctanoylglycerol (DiC_8; 1 mM), increased the P_o (Fig. 4). ATP and DiC_8 alone, cofactors required for kinase activity, had no effect on the P_o; however, omission of either cofactor or calcium from the bath (cytoplasmic solution) prevented PKC activation of the channel. Alkaline phosphatase (0.2 U/ml) added to the bath solution containing PKC partially reversed the PKC-induced rise in the P_o (P_o was 0.28±0.10 in control, 0.49±0.04 with PKC and 0.33±0.09 with alkaline phosphatase; $P < 0.05$; $n = 3$). Washing PKC from the bath

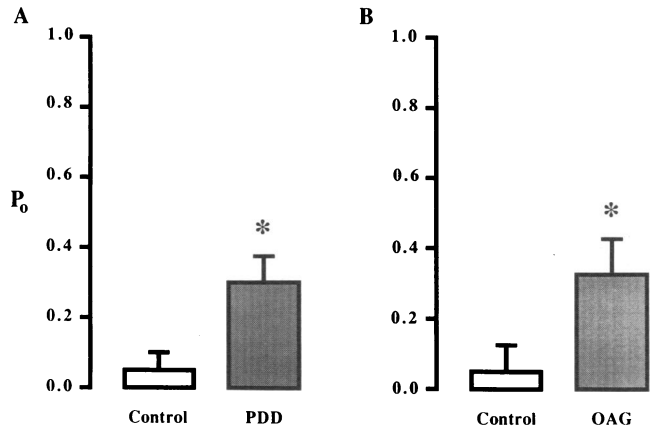

Figure 3. Activation of PKC by phorbol ester (PDD) or the DAG analogue OAG increased the P_o of the Cl^- channel. (*A*) PDD increased the P_o from 0.06±0.04 to 0.29±0.07 ($n = 7$; $P < 0.05$). (*B*) OAG increased the P_o from 0.06±0.05 to 0.33±0.09 ($n = 6$; $P < 0.05$). PDD and OAG increased the P_o within 2–3 min. The membrane voltage was hyperpolarized in cell-attached patches by 30 mV from the resting membrane potential.

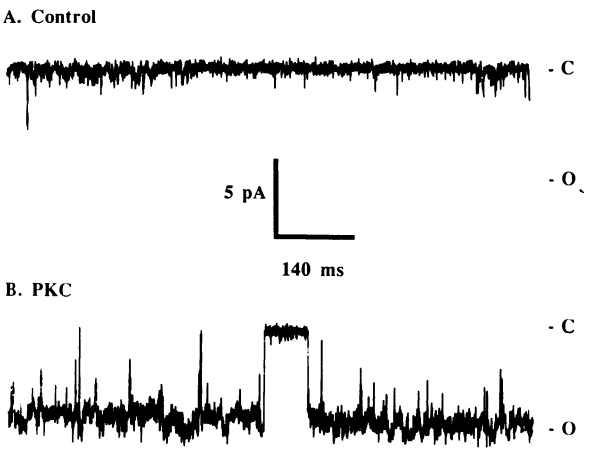

Figure 4. Representative current records illustrating the activation of the Cl^- channel by PKC in inside-out patches. The P_o was 0.10±0.06 in control and 0.48±0.10 after PKC (0.8 U/ml; $n = 8$; $P < 0.02$). PKC increased the P_o within 3 min. Experiments were performed with 100 nM Ca^{+2}, 1 mM ATP, 1 mM DiC_8, and 2 mM EGTA in the bath solution. The voltage across the membrane patch was −30 mV.

solution also reduced the P_o within 5–10 min, indicating that the inside-out membrane patches contained phosphatases. Taken together, these observations demonstrate that PKC activates the Cl channel.

In a previous study (10), we showed that the GTP-binding protein $G_{\alpha i\text{-}3}$ activates the 305-pS Cl^- channel in RCCT-28A cells. We tested the hypothesis that $G_{\alpha i\text{-}3}$ stimulation of the 305-pS Cl^- channel required active PKC. If the G protein activates the channel by a kinase-dependent mechanism, then inhibition of PKC by H8 should block G protein stimulation of the P_o. However, H8 (25 μM) did not prevent GTPγS, an analogue of GTP that activates the α subunit of G proteins, from increasing the P_o of the Cl^- channel (10). Thus, $G_{\alpha i\text{-}3}$ activation of the channel does not require PKC. We now test the hypothesis that PKC modulates the ability of $G_{\alpha i\text{-}3}$ to activate the channel. According to this hypothesis, pertussis toxin, an agent that uncouples G proteins from their receptors and thereby prevents G protein activation of effectors (10), should block the increase of the P_o by PKC. As illustrated in Fig. 5, pertussis toxin (PTX; 100 ng/ml) reduced the P_o to 0.4 After washout of PTX from the bath solution, addition of exogenous PKC to the bath solution did not increase the P_o of the Cl^- channel (Fig. 5). Subsequent addition of GTPγS (0.1 mM), which is known to reverse PTX inhibition (10), increased the P_o, indicating that channel "run-down" had not occurred. These observations suggest that PKC modulates the ability of $G_{\alpha i\text{-}3}$ to activate the Cl^- channel.

Effects of adenosine on diacylglycerol production. In a previous study (6), it was shown that CHA elevated inositol phosphate formation and increased intracellular calcium in RCCT-

4. In Fig. 5 it is evident that chloride channels were often active in inside-out patches (i.e., P_o was > 0) during the control period. The channels activated upon either formation of the inside-out patch configuration or by depolarization of the membrane. Results in all series of experiments were independent of the P_o in the control period. For example, in one typical experiment in which P_o in control was 0.19, PTX reduced P_o to 0 and PKC failed to increase P_o; however, GTPγS increased P_o to 0.39.

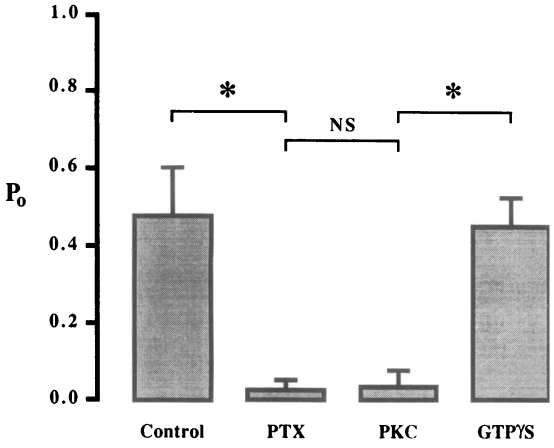

Figure 5. PKC does not activate the Cl^- channel after PTX treatment. The P_o's were as follows: control, 0.47±0.11; PTX (100 ng/ml; with NAD [1 mM] and ATP [1 mM]), 0.02±0.02; PKC (0.8 U/ml; with ATP (1 mM) and DiC_8 (1 μM), 100 nM Ca^{2+}), 0.03±0.02. GTPγS (0.1 mM) reversed PTX inactivation (P_o increased to 0.45±0.07). PTX inhibition occurred after 5 min whereupon the toxin was washed from the chamber. $*P < 0.05$. Paired experiments were performed on inside-out patches ($n = 5$). The voltage across the membrane patch was −30 mV.

28A cells, observations consistent with activation by CHA, via A_1 receptors, of phospholipase C. To provide independent support for this conclusion, we measured the generation of diacylglycerol in response to CHA. As illustrated in Fig. 6 A, CHA elicited time-dependent increases in DAG accumulation. Maximal stimulation was observed at 5 min after addition of 10^{-6} M CHA to the medium. DAG levels subsequently returned toward control levels. The concentration dependence of CHA-induced DAG formation at 5 min was also analyzed and the results are shown in Fig. 6 *B*. These data indicate that over the range of 10^{-7} to 10^{-5} M, i.e., concentrations that activated the Cl^- channel in cell attached patches (Fig. 1), CHA increased DAG accumulation in a concentration-dependent manner. Maximal stimulation was achieved at 10^{-5} M CHA; further increasing CHA concentrations had no additional stimulatory action. These observations show that adenosine increases DAG production in RCCT-28A cells.

Discussion

The major finding of the present study is that adenosine activates a 305 pS Cl^- channel in the apical cell membrane of rabbit RCCT-28A cells in culture by a membrane-delimited pathway involving an A_1 adenosine receptor, phospholipase C, DAG, PKC, and a G protein. This conclusion is supported by single-channel patch clamp recordings and by measurements of second messengers using biochemical approaches.

Adenosine stimulates the Cl^- channel by a membrane-delimited signal transduction pathway involving the A_1 receptor, phospholipase C, DAG, PKC, and a G protein. Four observations indicate that CHA stimulates the Cl^- channel by activating the A_1 adenosine receptor and not the A_2 receptor. First, at a concentration of 10^{-7} M, CHA binds specifically to A_1 receptors and does not increase cAMP levels, an A_2 receptor-mediated event (7). Second, CPX, a specific A_1 receptor antagonist at concentrations used here, completely prevents CHA activation of the channel. Third, CHA increased DAG production, an effect mediated by the A_1 adenosine receptor (1). Fourth, DDA, an inhibitor of adenylyl cyclase, did not alter the CHA-induced rise in the P_o. If CHA bound to the A_2 receptor, activated adenylyl cyclase, and increased the generation of cAMP, then it would be expected that DDA would block CHA activation of the channel.

Our patch clamp experiments show that A_1 receptors are located on the apical membrane of RCCT-28A cells. The presence of CHA in the patch pipette significantly increased the percentage of active Cl^- channels in cell-attached patches. The presence of adenosine receptors in the apical membrane of RCCT-28A cells, a cell line derived from rabbit CCD, is consistent with the observation by Husted et al. (33) that adenosine (A_2) receptors are also located on the apical membrane of rat inner medullary collecting duct cells. It is likely that adenosine receptors are also located on the basolateral membrane of CCD

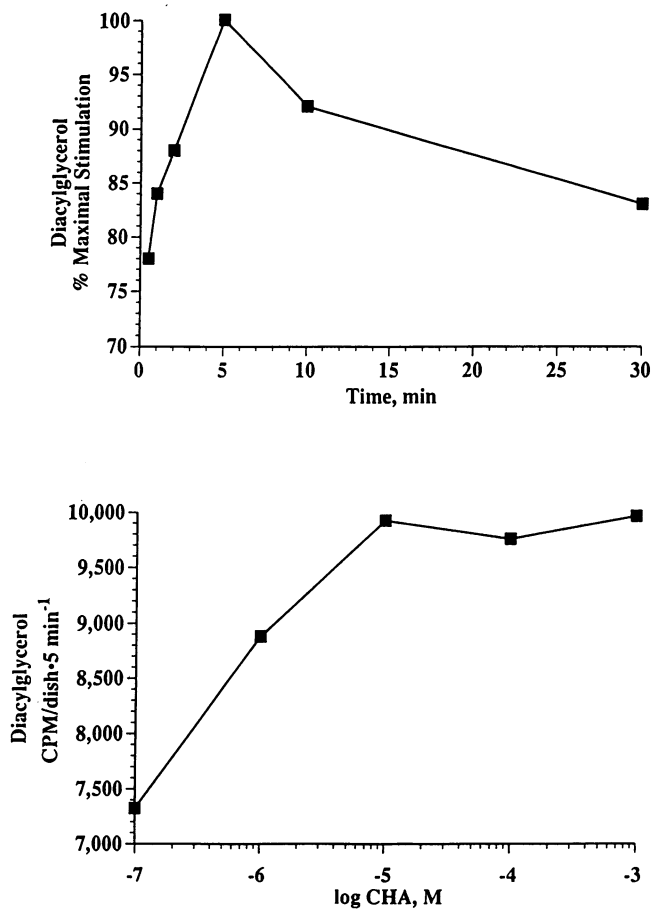

Figure 6. CHA increases DAG production. (*A*) DAG formation was measured following addition of 10^{-6} M CHA for the times indicated in the figure. Cells were labeled for either 3 or 24 h with [^{14}C]arachidonic acid. Duplicate analyses of DAG were performed as described in Methods. Results are expressed as percent of maximal stimulation for each experiment. 100% corresponds to an average DAG formation of 9,964±490 cpm ($n = 4$). (*B*) The concentration dependence of CHA stimulation of DAG formation was measured at 5-min intervals after addition of CHA at the indicated concentrations. Results represent the average of duplicate determinations of three experiments. DAG formation with vehicle alone was 4,398±1,128 cpm per dish/5 min.

and RCCT-28A cells. Because cells were grown on glass coverslips for the patch clamp experiments, the presence of adenosine receptors in the basolateral membrane could not be evaluated directly.

Several observations indicate that A_1 receptors stimulate phospholipase C in RCCT-28A cells and that such activation increases DAG production which, in turn, activates PKC and the Cl^- channel. First, A_1 receptor stimulation by CHA increases intracellular calcium and inositol phosphate formation in RCCT-28A and CCD cells (6, 8). Second, we now show that A_1 receptor activation increases the production of DAG. CHA elicited a rapid and dramatic increase in DAG production that coincided with the increase in the P_o of the channel. Moreover, the time course of CHA-induced DAG accumulation (Fig. 6 *A*) corresponds closely with the time course and concentration dependence of inositol phosphate formation evoked by 5'-*N*-ethylcarboxamidoadenosine (6). The fact that these adenosine analogues stimulated inositol phosphate and DAG formation with comparable kinetics suggests that DAG formation stimulated by adenosine analogues in RCCT-28A cells has its origins in inositol phospholipids and not in other phospholipids such as phosphatidylcholine (34, 35). This tentative conclusion is also consistent with the view that hydrolysis of phosphatidylcholine is thought to evoke sustained stimulation of DAG, whereas that derived from phosphatidylinositol bisphosphate results in a more rapid, but transient elevation of DAG levels (30, 34, 36). Unequivocal demonstration of this point, however, will require analysis of the fatty acid moieties of the DAG generated by these cells in response to adenosine. The second observation consistent with the view that adenosine analogues stimulate the channel by a pathway involving the A_1 receptor, phospholipase C, DAG, and PKC is that stimulation of PKC by OAG and PDD also increase the P_o of the Cl^- channel as did application of exogenous PKC. Because the adenylyl cyclase inhibitor DDA did not mimic the effect of CHA on the channel, nor did it influence CHA activation, we conclude that CHA did not activate the channel by an A_1 receptor pathway involving the inhibition of adenylyl cyclase and a reduction of cAMP. Taken together, these observations are most consistent with the conclusion that CHA activates the Cl^- channel by a sequential pathway involving A_1 adenosine receptor, phospholipase C, DAG, and PKC.

It should also be considered that adenosine may stimulate the Cl^- channel by increasing cell calcium levels. It is unlikely, however, that the rise in calcium directly activates the channel. First, CHA (10^{-6} M) increases cytosolic calcium by 65%, a change that does not increase the P_o of the 305-pS chloride channel in inside-out patches (9). Over the range of 100 nM to 1 μM calcium does not alter the P_o of the channel (9). Second, the increase in the P_o with CHA was similar to that elicited with exogenous PKC in inside-out patches, a situation in which the calcium concentration was held constant with EGTA (Fig. 4). Accordingly, it is unlikely that the rise in intracellular calcium directly increased the P_o of the Cl^- channel.

In a previous study we showed that $G_{\alpha i\text{-}3}$ located in the apical membrane of RCCT-28A cells activates the Cl^- channel (10). GTPγS and GTP increased the P_o of the channel in inside-out patches. Furthermore, PTX and GDPβS, an analogue of GDP that prevents G proteins from activating effectors (37, 38), decreased the P_o of the channel in inside-out patches (10). Because the protein kinase inhibitor H8 did not block GTPγS stimulation of the Cl^- channel, we concluded that G protein regulation of the channel did not require PKC (10). In the present study, we tested the hypothesis that PKC regulates the ability of the G protein to activate the Cl^- channel. We inhibited endogenous G proteins with PTX and then attempted to activate the channel with PKC. After PTX treatment, PKC failed to activate the channel, thereby indicating that PKC modulates the ability of $G_{\alpha i\text{-}3}$ to activate the Cl^- channel. In other cell types, protein kinases have been shown to modulate G protein regulation of their effectors (39–41). Our experiments do not allow us to determine whether PKC and the G protein regulate the channel by a sequential pathway (i.e., PKC phosphorylates $G_{\alpha i\text{-}3}$) or by independent pathways. For example, the G protein may facilitate the phosphorylation of the channel by PKC or, alternatively, the G protein may generate an important cofactor (such as arachidonic acid via activation of phospholipases) required for PKC activation of the channel. Additional experiments are required to examine these hypotheses.

G protein regulation of ion channels in epithelial cells and in electrically excitable cells has been shown to be either direct, indirect via activation of phospholipase A_2 and liberation of arachidonic acid or its metabolites, or to occur by both pathways (reviewed in reference 37). In this regard, DAG has been reported to stimulate phospholipase A_2 activity (42–44). This action of DAG may be mediated by direct binding to and activation of phospholipase A_2 and, at least in some cells (42), the activation is calcium dependent. We have not examined the mechanism of G protein regulation of the 305-pS Cl^- channel in RCCT-28A cells; elucidation of this mechanism is under investigation.

Adenosine regulates ion transport in a variety of epithelia, in addition to RCCT-28A cells, by pathways involving both A1 and A2 adenosine receptors. For example, adenosine stimulates sodium reabsorption in frog kidney A6 cells (45) and inhibits sodium reabsorption by inner medullary collecting duct cells in primary culture (33). Adenosine also enhances Cl^- secretion in airway epithelia (18), ileum (16, 46), and cornea (47) and inhibits Cl^- secretion in shark rectal gland (17). The second messenger of adenosine in these Cl^- secreting tissues, however, is cAMP, thus the effect is mediated via A_2 receptors (16–18). Adenosine stimulates Na^+-phosphate and Na^+-glucose transport in cultured opposum kidney cells by activating PKC and inhibiting protein kinase A (4). In electrically excitable cells, adenosine modulates calcium and potassium channel activity by a PTX-sensitive G protein (48, 49). It is thought that the G protein directly couples the adenosine receptor to the channels (48, 49). PTX also blocks adenosine activation of Cl^- channels in hippocampal neurons in culture (50) and prevents adenosine inhibition of N-type calcium channels in mouse sensory neurons in culture (51–53). Because PTX inhibits PKC regulation of the N-type calcium channel in sensory neurons (51), it is likely that PKC modulates the ability of a G protein to regulate calcium channels. Thus, adenosine regulates ion channels and transporter proteins in several cell types by a variety of pathways including protein kinases and G proteins.

Physiological role of the 305-pS Cl^- channel and its regulation by adenosine. We showed previously that the 305 pS Cl^- channel in the apical membrane of RCCT-28A cells is inactive in the basal state and is activated by cell swelling (14). Such activation of the Cl^- channel, accompanied by activation of a K^+ channel (E. Schwiebert and B. Stanton, unpublished observation), allows KCl and water to leave the cell thereby decreas-

ing cell volume. Thus, the Cl^- channel plays a key role in the regulatory volume decrease. Renal ischemia also causes cell swelling and the release of adenosine and 5'-AMP by cells of the CCD (1). Ecto-5'-nucleotidase located specifically on the apical membrane of rabbit intercalated cells dephosphorylates 5'-AMP thereby forming adenosine in the tubular fluid (1, 54). We postulate that during ischemia, adenosine is released and binds to A_1 receptors on the apical membrane of intercalated cells and activates the 305-pS Cl^- channel by a pathway involving phospholipase C, DAG, PKC, and a G protein. Activation of the Cl^- channel by adenosine causes cell volume to decrease. Adenosine may also act on adjacent principal cells and activate volume regulatory mechanisms. Thus, we propose that adenosine plays an important autocrine, and perhaps paracrine, role in cell volume regulation in the CCD during ischemia. Additional experiments, in progress, are required to test this hypothesis directly.

In summary, we report that adenosine activates a 305-pS Cl^- channel in the apical membrane of rabbit renal CCD cells (RCCT-28A) in culture by a membrane delimited pathway involving the A_1 adenosine receptor, phospholipase C, DAG, PKC, and a G protein. The Cl^- channel and this signaling pathway may play an important role in the regulatory volume decrease during renal ischemia.

Acknowledgments

We thank Dr. Bruce Koeppen for helpful discussions and comments on the manuscript and Ms. Susan Kennedy for expert technical assistance.

This study was conducted during the tenure of an Established Investigatorship of the American Heart Association (to Dr. Stanton) and was supported by National Institutes of Health grants DK-34533 (Dr. Stanton), DK-39654 (Dr. Spielman), and GM-34399 (Dr. Friedman). Mr. Schwiebert was supported by a National Institutes of Health predoctoral training fellowship (DK-07301).

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