Mechanism of Apical K^+ Channel Modulation in Principal Renal Tubule Cells

Effect of Inhibition of Basolateral Na^+-K^+-ATPase

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ABSTRACT The effects of inhibition of the basolateral Na^+-K^+-ATPase (pump) on the apical low-conductance K^+ channel of principal cells in rat cortical collecting duct (CCD) were studied with patch-clamp techniques. Inhibition of pump activity by removal of K^+ from the bath solution or addition of strophanthidin reversibly reduced K^+ channel activity in cell-attached patches to 36% of the control value. The effect of pump inhibition on K^+ channel activity was dependent on the presence of extracellular Ca^{2+}, since removal of Ca^{2+} in the bath solution abolished the inhibitory effect of 0 mM K^+ bath. The intracellular [Ca^{2+}] (measured with fura-2) was significantly increased, from 125 nM (control) to 335 nM (0 mM K^+ bath) or 408 nM (0.2 mM strophanthidin), during inhibition of pump activity. In contrast, cell pH decreased only moderately, from 7.45 to 7.35. Raising intracellular Ca^{2+} by addition of 2 μM ionomycin mimicked the effect of pump inhibition on K^+ channel activity. 0.1 mM amiloride also significantly reduced the inhibitory effect of the K^+ removal. Because the apical low-conductance K channel in inside-out patches is not sensitive to Ca^{2+} (Wang, W., A. Schwab, and G. Giebisch. 1990. *American Journal of Physiology.* 259:F494–F502), it is suggested that the inhibitory effect of Ca^{2+} is mediated by a Ca^{2+}-dependent signal transduction pathway. This view was supported in experiments in which application of 200 nM staurosporine, a potent inhibitor of Ca^{2+}-dependent protein kinase C (PKC), markedly diminished the effect of the pump inhibition on channel activity. We conclude that a Ca^{2+}-dependent protein kinase such as PKC plays a key role in the downregulation of apical low-conductance K^+ channel activity during inhibition of the basolateral Na^+-K^+-ATPase.

INTRODUCTION

The initial and cortical collecting duct (CCD) are the main sites for K^+ secretion in the kidney (Field and Giebisch, 1990; Wright and Giebisch, 1992). Two types of cells,

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principal and intercalated cells, are present in CCD (O'Neil and Hayhurst, 1985) and are known to serve different functions. Whereas intercalated cells reabsorb K^+ and secrete H^+ by mediation of an ATP-dependent K^+/H^+ exchanger (Wingo, Madsen, Smolka, and Tisher, 1990; Cheval, Barlet-Bas, Khadouri, Feraille, Marsy, and Doucet, 1991; Garg, 1991), principal cells are responsible for K^+ secretion and Na^+ reabsorption (O'Neil and Hayhurst, 1985; Palmer and Frindt, 1986). A large body of evidence suggests that an apical low-conductance K^+ channel (35 pS) is the main secretory K^+ channel (Frindt and Palmer, 1989; Wang, Schwab, and Giebisch, 1990; Ling, Hinton, and Eaton, 1991). This secretory K^+ channel has a high open probability, is pH sensitive, and is inhibited by Ba^{2+} but not TEA (Wang et al., 1990). Intracellular ATP has a dual effect: at high concentrations (1 mM) ATP inhibits the channel, whereas low concentrations (<0.1 mM) of ATP are essential for maintaining channel activity (Wang and Giebisch, 1991*b*). The latter stimulating effect of ATP on channel activity is mediated by phosphorylation of the channel by cAMP-dependent protein kinase A (PKA) (Wang and Giebisch, 1991*b*). In contrast to the effect of PKA, stimulation of protein kinase C (PKC) inhibits the K^+ channel by a process that has been shown to be dependent on the intracellular Ca^{2+} concentration (Wang and Giebisch, 1991*a*). Thus, the K^+ channel is modulated by two different second messenger pathways: the adenylate cyclase system increases channel open probability or recruits additional channels into the apical membrane, whereas stimulation of PKC reduces channel activity.

K^+ secretion in principal tubule cells is accomplished by two separate transport steps (Koeppen, Biagi, and Giebisch, 1983). First, K^+ is actively pumped into cells through the Na^+-K^+-ATPase. In a subsequent step, K^+ is secreted from cell into lumen through K^+ channels. Inhibition of the Na^+-K^+-ATPase activity by ouabain totally abolished K^+ secretion in the rabbit CCD (Nonaka, Warden, and Stokes, 1992) and it is likely that K^+ channel activity[1] is closely related to the turnover rate of the Na^+-K^+-ATPase. This study explores the mechanisms responsible for the sustained decrease of K^+ channel activity during inhibition of the Na^+-K^+-ATPase.

Some of the data were presented in abstract form at the 1991 Annual Meeting of the American Society of Nephrology (1991. *Journal of the American Society of Nephrology.* 2:733).

METHODS

Preparation of CCD Tubule

Pathogen-free Sprague-Dawley rats of either sex (80–120 g) (Taconic Farms Inc, Germantown, NY) were maintained on a normal rat chow or on a high (10%) potassium diet (Teklad Premier Laboratory Diets, Madison, WI) for 7–10 d. The latter diet increases the density of the K^+ channels. Since the properties of the K^+ channel of rats on a high potassium diet had been found to be identical to those obtained in tubules harvested from animals on a normal diet (Wang et al., 1990), we have pooled the data obtained from high potassium–adapted rats with those from control animals.

[1] In the following text, pump activity and K^+ channel activity refer to basolateral Na^+-K^+-ATPase and apical low-conductance K^+ channel activity, respectively.

Methods of tubule preparation were similar to those previously described (Wang et al., 1990). Cortical collecting tubules were dissected at room temperature in HEPES-buffered NaCl Ringer solution that contained (mM): 135 NaCl, 5 KCl, 1.8 $MgCl_2$, 1.8 $CaCl_2$, 5 glucose, and 10 HEPES (adjusted to pH 7.4 with NaOH) and transferred onto a 5 mm × 5 mm cover glass coated with "CELL-TAK" (Biopolymers, Inc., Farmington, CT) for immobilization. The cover glass was placed in a chamber mounted on the stage of an inverted microscope (model IM35; Carl Zeiss, Inc., Thornwood, NY) and the tubule was superfused with HEPES-buffered NaCl Ringer solution. Single tubules were cut open with a sharpened micropipette to expose the apical membrane. In this study only principal cells, visually identified, were used for patch-clamp studies. The criteria used for identification of principal cells have been described previously (Wang et al., 1990).

Experimental Media

Table I provides information on the composition of the bath solutions used in these experiments. The pipette solution contained (mM): 140 KCl, 1.8 $MgCl_2$, 1 EGTA, and 10 HEPES (pH 7.4). Ionomycin (Sigma Chemical Co., St. Louis, MO) and staurosporine (Boehringer Mannheim Corp., Indianapolis, IN) were dissolved in 50% DMSO solution. The concentrated stock solution was diluted by adding appropriate aliquots directly to the bath

TABLE I

Composition of Bath Solutions

No.	NaCl	KCl	$MgCl_2$	$CaCl_2$	EGTA	HEPES
			mM			
1	140	5	1.8	1.8	—	10
2	140	0	1.8	1.8	—	10
3	140	5	1.8	0	1	10
4	140	0	1.8	0	1	10

All solutions contained 5 mM glucose, and pH was 7.4.

chamber; the final concentration of DMSO in the bath was always $<0.5\%$, a concentration that had no effects on K^+ channel activity. All experiments were performed at 37°C.

Patch-Clamp Technique

Patch-clamp experiments were carried out by the methods described previously (Wang et al., 1990; Wang and Giebisch, 1991*b*). 1.65-mm-diam glass capillaries (Drummond Scientific Co., Broomall, PA) were used to pull patch-clamp electrodes. In the NaCl Ringer solution they had resistances of 4–6 MΩ when filled with 140 KCl. Channel activity was recorded with a model L/M-EPC7 patch-clamp amplifier (List Medical, Darmstadt, FRG) and currents were low-pass filtered at 1 KHz using an eight-pole Bessel filter (model 902LPF; Frequency Devices Inc., Haverhill, MA). The recordings were converted to digitized signals at a sampling rate of 44 KHz using a modified Sony PCM-501ES pulse code modulator and stored on videotape (Sony SL-2700). For analysis, the data were collected on an IBM-compatible AT hard disk at a rate of 4 kHz and analyzed using the pClamp software system (Axon Instruments, Inc., Foster City, CA) and SCAP (written by Dr. M. Hunter, University of Leeds, Leeds, UK). Channel activity was defined as the sum of the fractional open time (NP_o) at each channel level (from 1 to N), as determined by the equation:

$$NP_o = \sum_{n=1}^{N} (T_1 + T_2 + \ldots T_n)$$

where N was the number of visible current levels in the patch, and T_n the fractional open time spent at each of the observed current levels. NP_o in the control condition was calculated from data collected during a 2-min period. NP_o in experimental conditions was determined from 30-s data sampling periods every 60–120 s for a 10-min period after the decline of current amplitudes (as indication of pump inhibition). However, if the channel activity after pump inhibition had decreased to <20% of the control value for 15 s, we did not continue experiments and switched the bath to the control solution because cells became irreversibly damaged when pump inhibition was extended for longer time periods. The pipette holding potential was usually made more positive (hyperpolarization) after pump inhibition than that in the control condition for better resolution of K^+ current transition. Since P_o of the low-conductance K^+ channel is not voltage dependent, the change of the holding potential had no effect on channel P_o. The K^+ channel has a high P_o (>0.9) and multiple channels are frequently observed in patches. Accordingly, it is very difficulty to identify the closed current level in cell-attached patches unless channel activity is inhibited by some intervention. However, the channel closed state can be determined by application 2 mM ATP or 0.5 mM Ba^{2+} in inside-out patches that are excised in the solution containing 10 U of PKA and 0.1 mM MgATP, as PKA and ATP can prevent channel rundown (Wang and Giebisch, 1991*b*). Therefore, this method was used to identify the closed state of the channel if zero current level could not be determined in cell-attached patches.

Cell pH and Ca^{2+} Measurement

Intracellular pH was measured by methods described previously (Geibel, Giebisch, and Boron, 1990). For these measurements of cell pH and of Ca^{2+}, a tubule area was selected that consisted of CCD principal cells (two to three cells). For Ca^{2+} measurements, cells were incubated with fura-2-AM (5 μM) at room temperature for 10 min. After dye loading, tubules were washed twice using the control solution and transferred to a microperfusion chamber. A fluorescence imaging system was used to measure Ca^{2+}. The dye in the specimen was excited with light of the desired wavelengths (340–380 nM) using a 150-W xenon source coupled to two monochromators with variable band width. The light exiting the monochromators entered a computer-controlled high speed electronic shutter that allowed incident light to alternate between two excitation wavelengths. Even illumination of the entire field of cells was achieved by an arrangement that permitted the aperture of the lens to be filled by the light channeled via a bifurcated bundle of quartz fibers. The fluorescent signal was sampled with an intensifying (Videoscope KS-1380) video camera (model 72; Dage-MIT, Inc., Michigan City, IN). The Ca^{2+}-bound fluorescent and unbound fura-2 were analyzed by rapidly alternating the excitation wavelength between 340 and 380 nm and measuring the emission signals electronically at 512 nm. For each experimental protocol, image signals (ratio 340 nm/380 nm) were both collected and displayed on a video monitor at a maximum rate of 15 ratio pairs/s and simultaneously recorded on an optical memory disc recorder. Areas where principal cells were clustered were selected and the ratio-metric information within a field was displayed on the computer monitor and stored on the hard disk. The original traces are averages of two to three cells analyzed from the same experiment. After each experiment the maximal fluorescence ratio (R_{max}) and the minimal fluorescence ratio (R_{min}) were determined using 5 μM ionomycin or 2 mM $MnCl_2$, respectively (Grynkiewicz, Ponie, and Tsien, 1985). Intracellular calcium concentrations were calculated using the equation described by Grynkiewicz et al. (1985):

$$[Ca^{2+}] = [(R - R_{min})/(R_{max} - R)] \times (F_{max}/F_{min}) \times K_d$$

where F_{max} is the fluorescence at 380 nm at 0 mM Ca^{2+} bath solution, F_{min} is the fluorescence at saturating calcium, and (K_D) is a dissociation constant (224 nM for the fura-2-Ca^{2+} complex). Control experiments were carried out to exclude the effect induced by DMSO.

Statistics

Data are presented as mean ± SEM. Where appropriate, Student's *t* tests for paired and unpaired data were used to assess significance of difference.

RESULTS

Effect of Pump Inhibition on Apical K^+ Channel

Fig. 1 shows a representative recording in which channel activity was monitored in cell-attached patches. It is apparent that during control conditions eight current levels are visible. Since channel opening and closure are likely to follow binomial

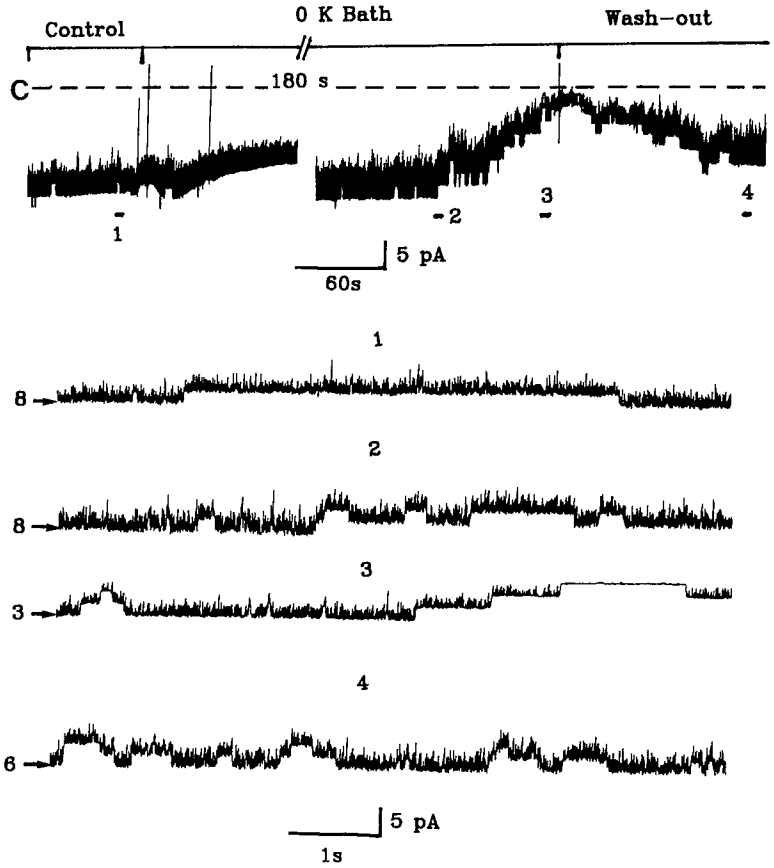

FIGURE 1. Effect of 0 mM K^+ bath solution (solution 2) on apical low-conductance K^+ channel activity. Channel activity was recorded in a cell-attached patch. The pipette holding potential was −25 mV (control) and 0 mV (0 mM K^+ and wash-out), respectively. Recordings are shown at slow and fast time courses. The number of current levels of the K^+ channels are indicated on the left of the figure and channel closed state is indicated by a dotted line and labeled *C*. Four parts of recording, indicated by a short bar and numbered *1, 2, 3,* and *4* in the upper tracing, are displayed at fast time course in the lower part of the figure.

distribution, the channel number and P_o are calculated using the equation (Colquhoun and Hawkes, 1983)

$$P(n) = N!/n!(N - n)![P_o^n(1 - P_o)^{N-n}]$$

where $P(n)$ is the probability of simultaneous opening of n channels, N is the number of functional channel in the patch, and $n = 1, 2, \ldots N$. From the measured fractional open time in the eighth current level (90%; not shown), the best fit is obtained with eight channels with a P_o of 0.98. These data are consistent with previous results (Wang et al., 1990).

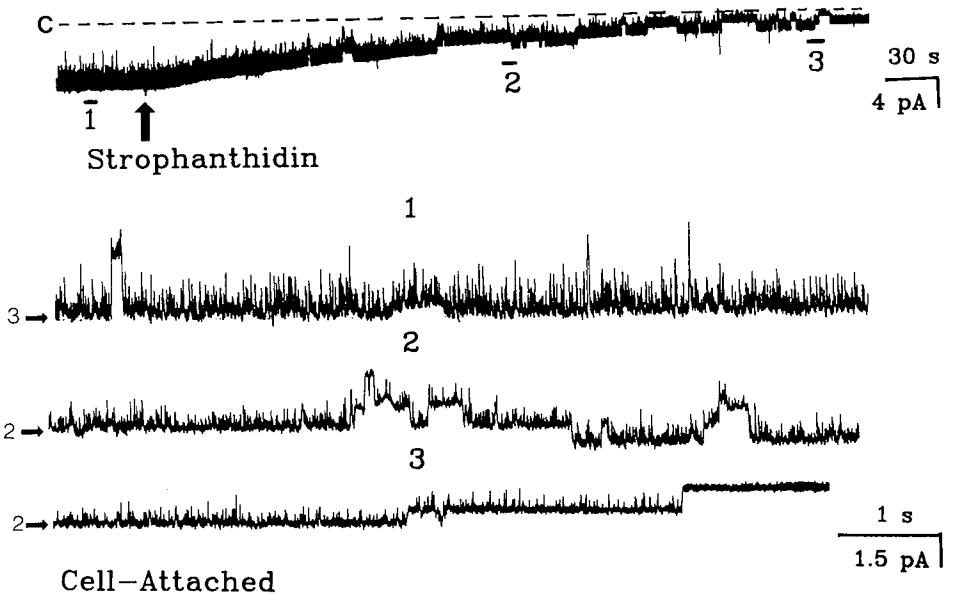

FIGURE 2. Effect of 0.2 mM strophanthidin on apical low-conductance K^+ channel activity. Channel activity was recorded in a cell-attached patch. The pipette holding potential was 0 mV. The number of current levels of the K^+ channels is indicated on the left of the figure and channel closed state is indicated by a dotted line and labeled *C*. Three parts of recording indicated by a short bar and numbered *1*, *2*, and *3* in the upper tracing are displayed at fast time course in the lower part of the figure.

Inhibition of the Na^+-K^+-ATPase by lowering the $[K^+]$ bath to 0 mM decreased the channel current amplitude owing to the decline of the driving force for K^+ without instantaneously changing NP_o. However, channel activity started to decline progressively in this membrane patch 5–6 min after reducing the bath $[K^+]$ and, over the next 10 min, decreased to 30% of the control value. The inhibitory effect of exposure of principal cells to the 0 mM K^+ bath solution was reversible. Inspection of Fig. 1 shows that returning to the initial 5 mM K^+ bath solution almost fully restored channel activity.

Similar results on K^+ channels are also observed when 0.2 mM strophanthidin was used to inhibit Na^+-K^+-ATPase. Fig. 2 shows a representative experiment. In the

control condition 3 K^+ channels were present with a P_o of 0.95. Application of strophanthidin not only decreased the current amplitude but also reduced NP_o progressively to 32% of the control value.

We conclude from these experiments that inhibition of Na^+-K^+-ATPase reduces the activity of the K^+ channel. Studies in which the time course of channel activity was monitored after restoration of pump activity show that the inhibition of apical channels is reversible.

Effect of Basolateral Pump Inhibition on Cell pH

The K^+ channel of principal tubule cells is highly pH sensitive and has been shown to be inhibited by acidification of the cytosolic medium (Wang et al., 1990). It is possible that blocking the Na^+-K^+-ATPase reduces intracellular pH, and that the decrease of K^+ channel activity is mediated by changes of the cell pH. Cell pH changes could be mediated by the increase of cell Na^+ after inhibition of pump activity and diminished H^+ extrusion by Na^+/H^+ exchange (Chaillet, Lopes, and Boron, 1985), since the electrochemical driving force for H^+ extrusion would be compromised. Accordingly, cell pH was measured in single principal cells under control conditions and after exposure of the single tubules to a K^+-free medium. These pH measurements are summarized in Table II. It can be seen that intracellular pH was only moderately

TABLE II

Summary of Intracellular pH under Control Conditions and during Superfusion with 0 mM K

	Control	0 mM K^+ + Ca^{2+}	0 mM K^+ (Ca^{2+} free)
pH	7.45 ± 0.03	7.35 ± 0.02*	7.35 ± 0.02*
N	15	10	5

Values are mean ± SEM and *N* is the number of observation. *$P < 0.05$.

decreased, from 7.45 ± 0.03 to 7.35 ± 0.02, whether Ca^{2+} was present or not. Absence of marked pH changes in principal cells after basolateral pump inhibition was also reported by Silver, Frindt, Windhager, and Palmer (1993).

Effect of Basolateral Pump Inhibition on Cell Ca^{2+}

Inhibition of the Na^+-K^+-ATPase in renal proximal tubule cells has been shown to increase cell Ca^{2+} concentration (Wang, Messner, Oberleithner, Lang, and Deetjen, 1984; Yang, Lee, and Windhager, 1988). Fig. 3 *A* displays a continuous recording of Ca^{2+} measurements in CCD principal cells before and after inhibition of the basolateral Na^+-K^+-ATPase using 0 mM K^+. Intracellular Ca^{2+} rose significantly to 335 ± 30 nM but returned to control values (122 ± 3 nM) when basolateral pump activity was restored. Similar results were also obtained when Na^+ pump activity was inhibited by application of 0.2 mM strophanthidin. Fig. 3 *B* shows a representative tracing of the time course of cell Ca^{2+} alterations. Application of strophanthidin increased reversibly intracellular Ca^{2+} from 125 ± 5 to 408 ± 40 nM. Fig. 4 depicts the time course of cell Ca^{2+} concentration changes and channel activity. Data were normalized to facilitate comparison. It is apparent that the increase in Ca^{2+}

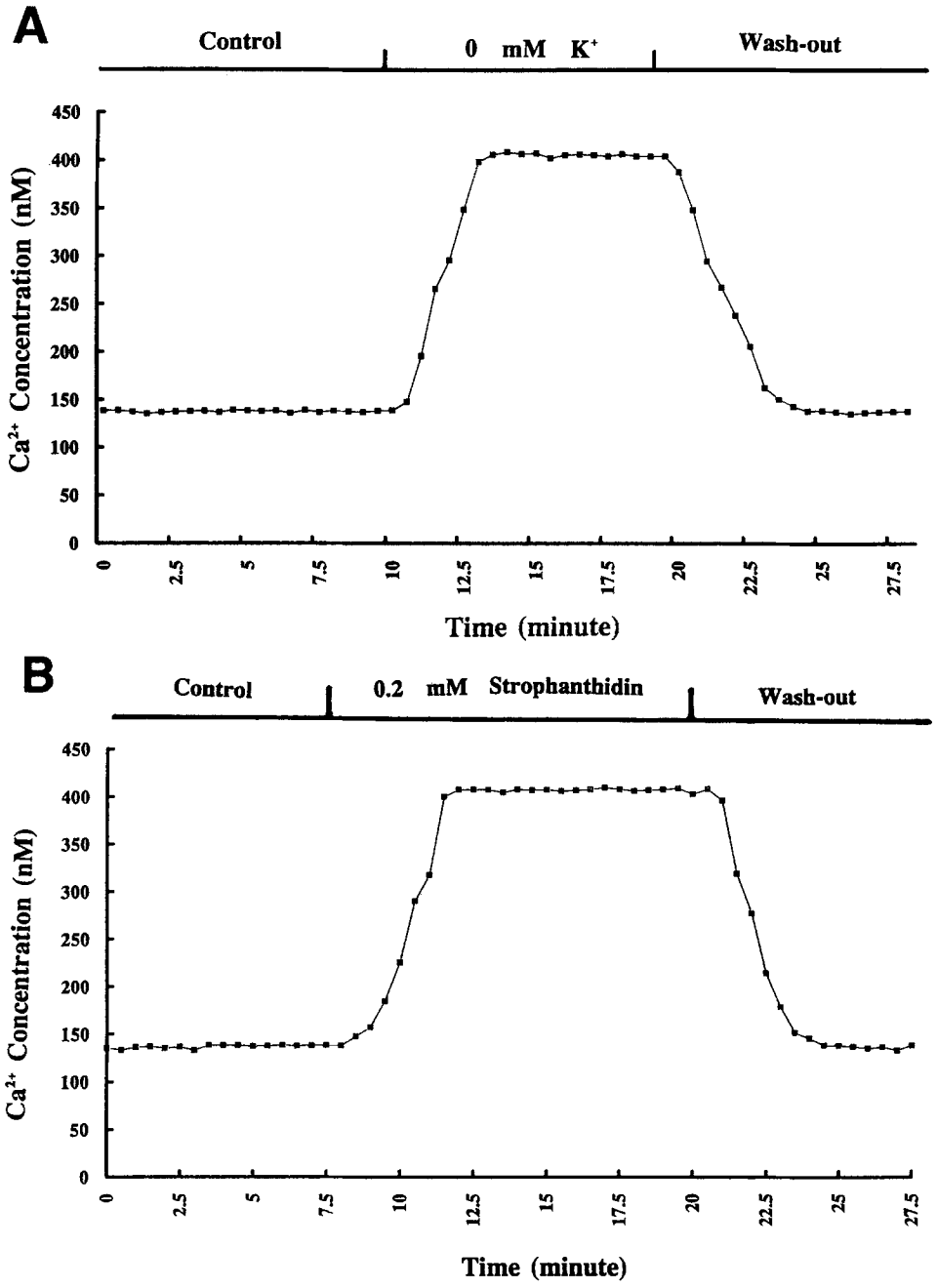

FIGURE 3. Representative experiment of the time course of intracellular Ca^{2+} concentration changes in CCD principal cells after switching bath solution from the control Ringer (solution 1) to 0 mM K^+ solution (*A*) or 0.2 mM strophanthidin-containing solution (*B*).

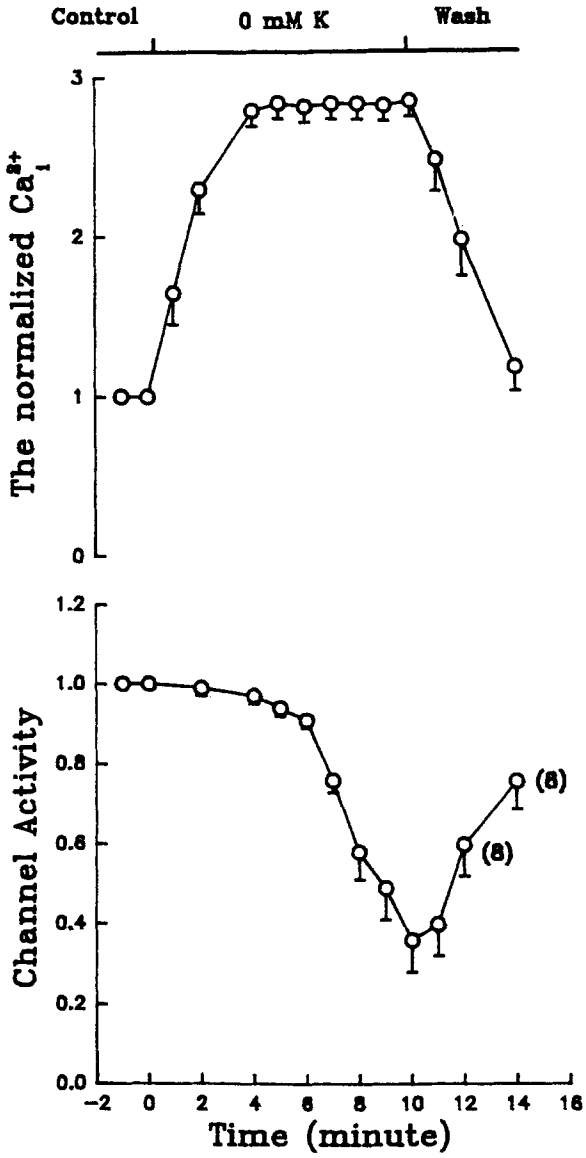

FIGURE 4. The time course between the normalized cell Ca^{2+} concentration (Ca_i^{2+}) and channel activity. The observation number for channel activity is 15, or as indicated in the figure, and the experimental number for Ca_i^{2+} is 9.

concentration occurs faster than the decrease of channel activity. Whereas the peak plateau of Ca^{2+} is reached within 4 min, channel activity is not significantly reduced until ~6 min. This delay suggests that the Ca^{2+} effect on channel activity is indirect.

We examined the role of intracellular Ca^{2+} on the down regulation of apical K channel activity in additional experiments in which the effects of changes in cytosolic Ca^{2+} on apical K^+ channel activity were tested while basolateral pump activity was left intact. For these experiments, ionomycin was used to clamp the intracellular Ca^{2+} concentration (see Table III). Fig. 5 shows that in the absence of the extracellular

TABLE III

Summary of Intracellular Ca^{2+} Concentrations in the Control Conditions and during Experimental Conditions

	Control	0 mM K	Strophanthidin	Amiloride + 0 K	Ionomycin
C, nM	125 ± 5	335 ± 30*	408 ± 40*	220 ± 15*	338 ± 17*
N	26	9	4	8	5

Values are mean ± SEM; *C* is the concentration and *N* is the number of observations. *$P < 0.001$. In experiments in which ionomycin was used, the extracellular Ca^{2+} was chelated with 1 mM EGTA to 330–340 nM.

Ca^{2+}, ionomycin has no significant effect on channel activity. However, adding 330 nM Ca^{2+} to the bath in the presence of ionomycin progressively decreased channel activity in these cell-attached patches in a manner similar to that observed during exposure of principal cells to the 0 mM K^+ bath solution. The Ca^{2+}-mediated inhibition of channel activity was fully reversible. Fig. 5 shows that removal of the extracellular Ca^{2+} restored channel activity to control levels.

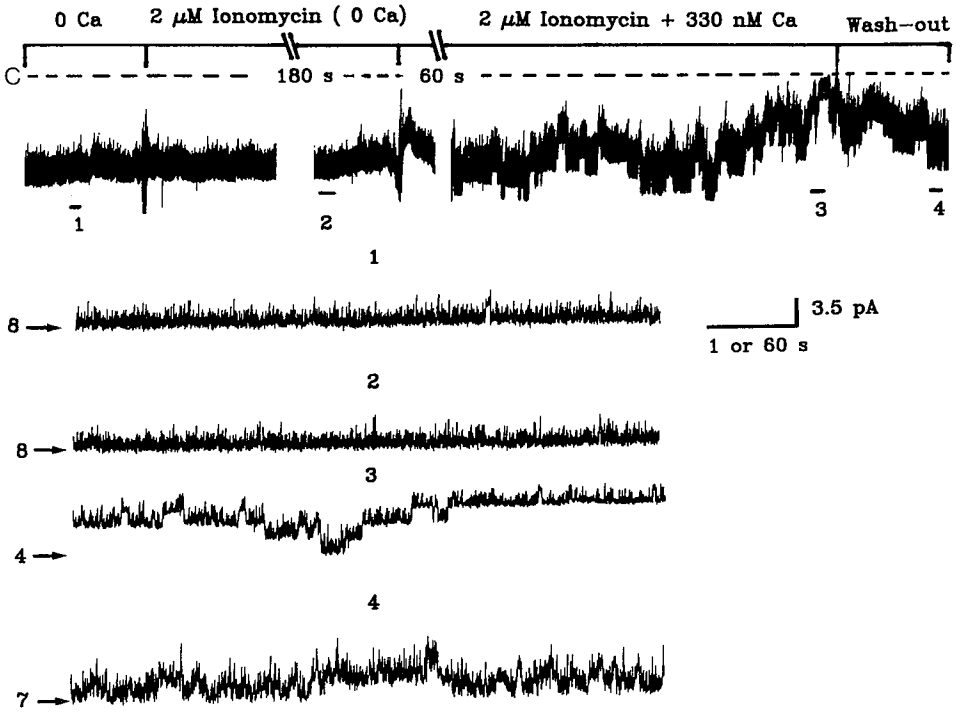

FIGURE 5. Effect of 2 μM ionomycin on apical low-conductance K^+ channel activity of CCD principal cells. The channel recording was made in a cell-attached patch. The pipette holding potential was 0 mV (Ca^{2+}-free period) and was increased to 30 mV after the addition of 330 nM Ca^{2+}. The number of current levels of the K^+ channels is indicated on the left of the figure and channel closed state is indicated by a dotted line and labeled *C*. Four parts of recording indicated by a short bar and numbered *1, 2, 3,* and *4* in the upper tracing are displayed at fast time course in the lower part of the figure.

Fig. 6 summarizes results obtained from the ionomycin experiments in which cell Ca^{2+} was clamped to different levels. It demonstrates a significant relationship between intracellular Ca^{2+} and K^+ channel activity. K^+ channel activity was reduced to 29 ± 4% of the control value (n = 3) by raising intracellular Ca^{2+} to 330 nM, the mean value of the cell Ca^{2+} concentration during inhibition of the pump. Channel activity was further reduced by raising intracellular Ca^{2+} concentration and completely blocked by 1 μM.

The important role of the intracellular Ca^{2+} concentration in modulating K^+ channel activity was further confirmed by experiments in which removal of the extracellular Ca^{2+} was shown to abolish the inhibitory effect of the 0 mM K^+ bath solution. These data are summarized in Fig. 7, in which the time course of channel activity is monitored during a 10-min period after basolateral pump inhibition by exposure of CCD to 0 mM K^+. In the presence of extracellular Ca^{2+}, channel activity

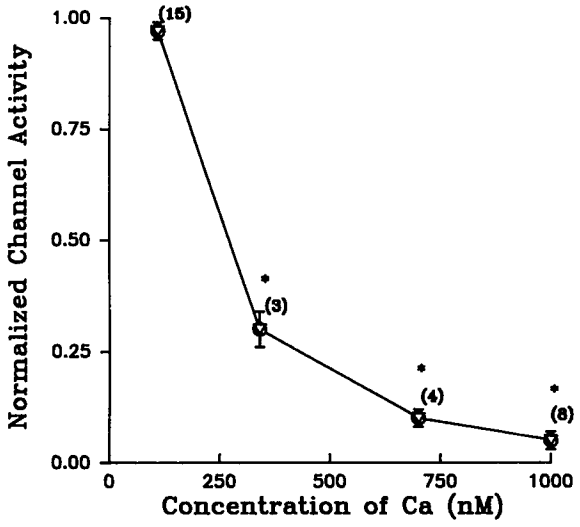

FIGURE 6. The effect of the ionomycin-induced increase of Ca^{2+} concentration on apical low-conductance K^+ channel activity. The percentage of inhibition of channel activity was calculated by the equation: $1\text{-}NP_e/NP_o$, where NP_e and NP_o are mean channel activity in experimental and control conditions, respectively. * indicates that results are significantly ($P < 0.001$) different from the control group in which ionomycin was used in the absence of extracellular Ca^{2+}.

starts to decline significantly 6 min after inhibition of the pump. Channel activity was reduced to 36 ± 7% (see Table IV) of the control value within 10 min after the inhibition of pump activity by removal of K^+ (n = 15) or by addition of 0.2 mM strophanthidin (n = 3). In sharp contrast, K^+ channel activity was fully sustained at control levels in the absence of extracellular Ca^{2+}, despite inhibition of Na^+-K^+-ATPase activity (see Table IV).

The strong Ca^{2+} dependence of the effect of basolateral pump inhibition on apical K^+ channels suggests that the increase of intracellular Ca^{2+} after reduction of pump turnover results from decreased efflux of intracellular Ca^{2+} or increased influx of extracellular Ca^{2+}. Inhibition of basolateral ATPase by exposure to a low potassium medium results in an increase of cell Na^+ and decreased extrusion of Ca^{2+} through the basolateral Na^+/Ca^{2+} exchanger which has been identified in CCD cells by several investigators (Taniguchi, Marchetti, and Morel, 1989; Bourdeau and Lau,

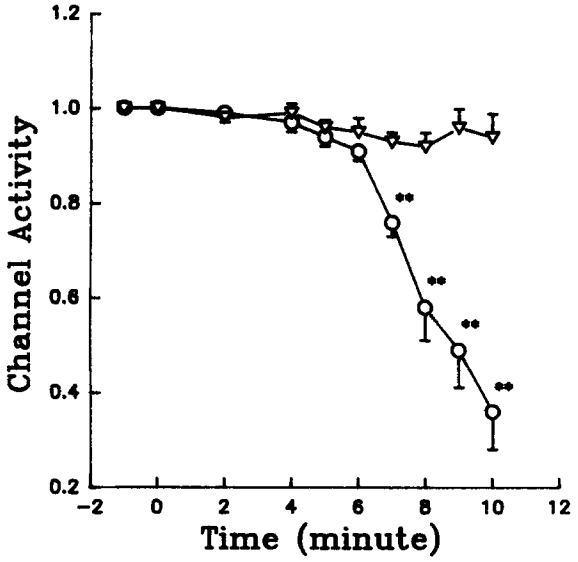

FIGURE 7. Time course of channel activity after inhibition of Na^+-K^+ pump by 0 mM K^+ bath in the presence of Ca^{2+} (*circles*, $n = 15$) and in the absence of Ca^{2+} (*triangles*, $n = 8$). The time at which the K^+ channel current amplitude declines is considered as 0 min. ** indicates that data are significantly (P at least < 0.05) different from results obtained in the absence of Ca^{2+} at the corresponding time point and from their control value.

1990; Frindt and Windhager, 1990). Two mechanisms could cause an increase of Ca^{2+} influx. First, cell depolarization by pump blockade could activate voltage-sensitive Ca^{2+} channels and thus elevate intracellular Ca^{2+} concentration. Second, inhibition of Na^+ pump turnover rate and the attendant rise of cell Na^+ (Natke and Stoner, 1982; Horisberger and Giebisch, 1989) may result in the operation of the Na^+/Ca^{2+} exchanger in the reversed direction (Yang et al., 1988). As a consequence, extracellular Ca^{2+} could enter the cell and be responsible for the sharp increase of cell Ca^{2+}. The first possibility is unlikely to play an important role in mediating the cell Ca^{2+} changes, because no significant cell Ca^{2+} alterations were observed when the cell membrane potential was depolarized by 40 mM K^+ (Wang, W., unpublished observations). Thus, Ca^{2+} influx is probably mediated by reversed turnover of the Na^+/Ca^{2+} exchanger.

The dependence of the rise of cell Ca^{2+} on changes of Na^+ transport was also explored in experiments in which amiloride was used to minimize cell sodium

TABLE IV

Normalized Channel Activity 10 min after Inhibition of Na^+-K^+-ATPase

Group A	Group B	Group C	Group D
36 ± 7%*	94 ± 5%‡	75 ± 5%*‡	63 ± 8%*‡
$n = 18$	$n = 8$	$n = 6$	$n = 5$

Cells in group A were superfused with Ca^{2+}-containing 0 mM K^+ solution (solution 2, $n = 15$) or 0.2 mM strophanthidin ($n = 3$) during the experimental period. Cells in group B were bathed in a Ca^{2+}-free 0 mM K^+ solution (solution 4). Cells in groups C and D were superfused with solution 2. 200 nM staurosporine was used in group C and 0.1 mM amiloride in group D. Values are mean ± SEM and N is number of observations. *Data are significantly ($P < 0.01$) different from the corresponding control. ‡Data are significantly ($P < 0.01$) different from data of group A.

concentration changes after basolateral pump inhibition. Although application of 0.1 mM amiloride did not completely block the increase of intracellular Ca^{2+} induced by the 0 mM K^+ bath solution, the magnitude of the change of cell Ca^{2+} (Ca^{2+} rose to 220 ± 15 nM in the presence of amiloride) was significantly lower than the concentration of 335 ± 30 nM in the absence of amiloride (see Table III). In addition, amiloride application also attenuated the effect of the 0 mM K^+ bath solution on apical channel activity. Fig. 8 displays the time course of the decrease of K^+ channel activity after inhibition of Na^+-K^+-ATPase with 0 mM K^+ bath in the presence or absence of 0.1 mM amiloride. Reduction of channel activity in the presence of amiloride was significantly less than without amiloride (see Table IV). These results are consistent with the interpretation that the effect of inhibition of Na^+-K^+-ATPase on apical K^+ channel activity is mediated by a Ca^{2+} signal related to modulation of basolateral Na^+/Ca^{2+} exchange.[2]

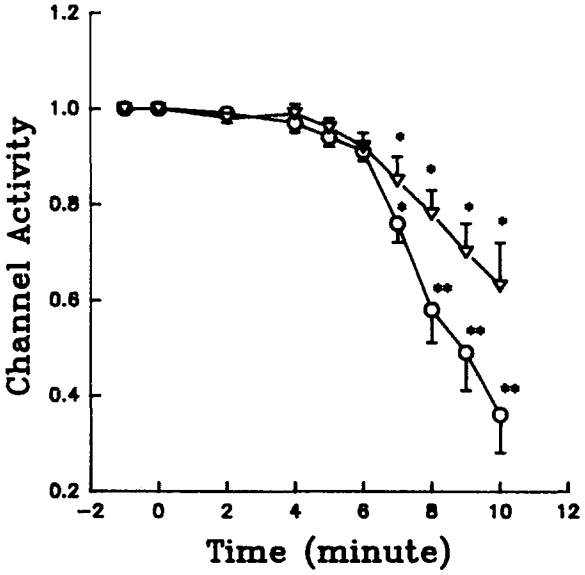

FIGURE 8. Time course of channel activity after inhibition of Na^+-K^+ pump by 0 mM K^+ bath in the presence of amiloride (*triangles*, $n = 5$) or in the absence of 0.1 mM amiloride (*circles*, $n = 15$). The time at which the K^+ channel current amplitude declines is considered as 0 min. * indicates that data are significantly (P at least <0.05) different from their control value. ** indicates that data are significantly (P at least <0.05) different from results obtained in the presence of amiloride at the corresponding time point and from their control value.

The Role of Ca^{2+}-dependent Protein Kinase

Previous studies of inside-out patches showed that the apical K^+ channel was not sensitive to Ca^{2+} (Wang et al., 1990). Thus, the present observation that an increase of the intracellular Ca^{2+} inhibits channel activity suggests that the observed inhibitory

[2] The observation that amiloride did not completely prevent the increase in cell Ca^{2+} after basolateral Na^+-K^+ pump inhibition is consistent with the view that apical channel-mediated Na^+ entry is not the sole mechanism by which Na^+ ions enter principal tubule cells. It has been shown in an electron probe study of isolated perfused CCD that the increase of cell Na^+ after basolateral ATPase inhibition can be partially prevented by application of luminal amiloride (Sauer, Flemmer, Thurau, and Beck, 1990). Only the removal of basolateral Na^+ in the presence of luminal amiloride was able to prevent the rise of cell Na^+ after basolateral pump inhibition. The putative Na^+ entry pathways could include unselective cation channels and/or incompletely blocked Na^+/H exchange.

effect of the elevated cytosolic Ca^{2+} levels must be indirect, possibly the result of changes in the activity of Ca^{2+}-dependent second messengers such as Ca^{2+}-dependent PKC. Operation of such a mechanism appears possible because we demonstrated that stimulation of PKC inhibited the K^+ channel and that this effect was enhanced by raising Ca^{2+} (Wang and Giebisch, 1991*a*). We have now used staurosporine to inhibit PKC (Ederveen, Van Emst-De Vries, De Pont, and Willems, 1990) to test the hypothesis that changes in PKC activity mediated the reduction of channel activity.

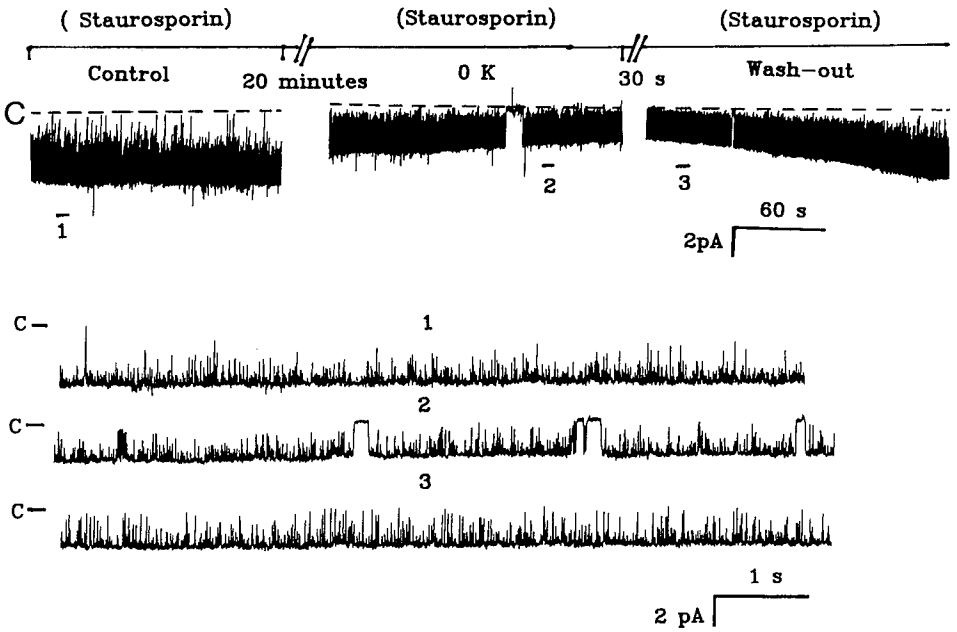

FIGURE 9. Effect of 0 mM K^+ bath solution on apical K^+ channel activity in the presence of 200 nM staurosporine. The channel recording was made in a cell-attached patch. The pipette holding potential was 0 mV (control) and 30 mV (0 mM K^+ and wash-out), respectively. Channel closed state is indicated by a dotted line and labeled *C*. Three parts of recording indicated by a short bar and numbered *1*, *2*, and *3* in the upper tracing are displayed at fast time course in the lower part of the figure.

Fig. 9 summarizes the results of an experiment in which the effect of staurosporin was explored. Application of 200 nM staurosporine had no effect on K^+ channel activity in cell-attached patches (data not shown). However, staurosporine significantly attenuated the inhibitory effect of the 0 mM K^+ bath solution. Fig. 9 shows that in contrast to the marked decline otherwise produced by inhibition of Na^+-K^+-ATPase activity, channel activity was only moderately decreased by removal of K^+ from the bath when the PKC inhibitor was present. The right-hand part of Fig. 9 also demonstrates that after restoration of K^+ the amplitude of the K^+ channel current

increased progressively. The increase of current amplitude indicated that cell membrane potential is repolarized after restoration of pump activity.

Fig. 10 summarizes the time course of channel activity in control and staurosporin-treated tubules. Results are from six experiments in which staurosporine was used. It is apparent that application of this PKC blocker reduced the effect of pump inhibition. Channel activity was reduced to only 75 ± 5% of the control value in the presence of staurosporine, whereas a much larger reduction of channel activity, to 36 ± 7% of the control values (see Table IV), was observed in the presence of an intact PKC system. These results indicate that Ca^{2+}-dependent signal transduction pathways such as PKC play a key role in the coupling mechanism between reduced

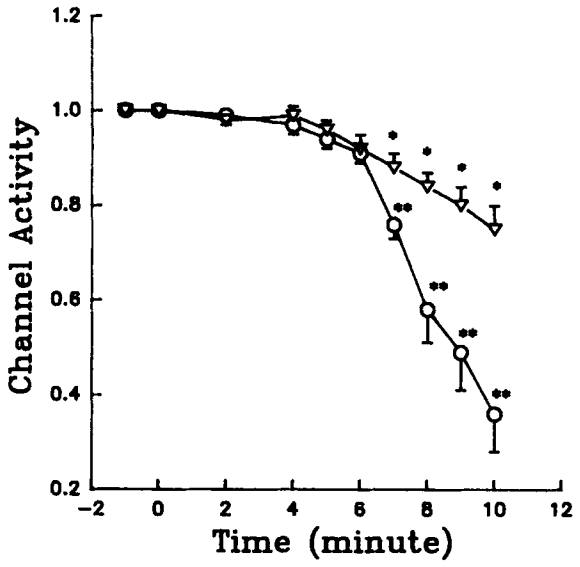

FIGURE 10. Time course of changing channel activity after inhibition of Na^+-K^+ pump using 0 mM K^+ bath solution in the presence of staurosporine (*triangles*, $n = 6$) or in the absence of 200 nM staurosporine (*circles*, $n = 15$). The decline of K^+ channel current amplitude is considered as 0 min. * indicates that data are significantly (P at least <0.05) different from their control value. ** indicates that data are significantly (P at least <0.05) different from results obtained in the presence of staurosporine at the corresponding time point and from their control value.

basolateral Na^+-K^+-ATPase turnover rate and downregulation of apical K^+ channel activity.

DISCUSSION

Coupling of Apical K^+ Channel Activity to Basolateral Na^+-K^+-ATPase Turnover: Effect of Pump Inhibition

Ion transport in epithelia through transcellular pathways involves solute translocation across two barriers, the apical and basolateral cell membrane. To maintain electrolyte concentration and volume in the presence of varying rates of transcellular ion transport, the passive movement of ions across the apical membrane must be closely coordinated with active ion flux rates across the basolateral cell membrane (Schultz, 1981). In CCDs, the main routes for K^+ entering and leaving principal cells are through the basolateral Na^+-K^+-ATPase and the low-conductance apical K^+ channel, respectively (Stanton and Giebisch, 1992; Wang, Sackin, and Giebisch, 1992; Wright

and Giebisch, 1992). In this study we offer evidence that apical K^+ channel activity is linked to basolateral pump turnover rate: inhibition of the basolateral Na^+-K^+-ATPase significantly lowered channel activity. The experimental conditions of this study involved a marked reduction of pump activity by deletion of extracellular K^+ or exposure to the cardiac glycoside strophanthidin. These interventions induce a significant rise of Na^+ and a fall of K^+ in CCD cells, a prompt depolarization of the membrane potential, and significant reduction of the potassium conductance (Natke and Stoner, 1982; Stokes, 1984; Horisberger and Giebisch, 1989). There is also a large body of experimental evidence based on experiments in other epithelia (Schultz, 1981; Lang and Rehwald, 1992) that active, ATP-mediated Na^+ exchange is tightly coupled to the potassium conductance of the basolateral membrane, the site of active Na^+-K^+ exchange.

Several mechanisms have been proposed to identify the mediator linking pump with K channel activity. These include changes in intracellular pH, Ca^{2+}, the ATP/ADP ratio, and the cell membrane potential. Since apical K^+ channel activity is not voltage dependent, and since it is also observed that depolarization does not alter NP_o, the possibility that the downregulation of channel activity is mediated by depolarization can be excluded. The downregulation of K^+ channel activity after the reduction of Na^+-K^+-ATPase activity observed in our experiments is accompanied by a significant elevation of cytosolic Ca^{2+} and was shown to involve activation of PKC. However, the possible role of other factors contributing to the modulation of K^+ channels when ATPase activity is reduced has to be considered.

Two arguments make it unlikely that cell pH changes play a major role in the downregulation of apical K^+ channel activity observed under the present experimental conditions. First, the modest decrease of 0.1 pH unit, caused by inhibition of the basolateral pump, can at best account for only a 20% reduction of channel activity, even when extrapolating from the steepest slope of the pH–response curve obtained in previous studies (Wang et al., 1990). Second, the removal of extracellular Ca^{2+} almost completely abolished the inhibitory effect of the 0 mM K^+ bath solution on K^+ channel activity, although the magnitude of the pH change was the same as that in Ca^{2+}-containing Ringer. Thus, we can exclude intracellular pH as a major component in mediating the effect of inhibition of pump activity on apical K^+ channel activity of the rat CCD.

Another potential candidate for K^+ channel modulation is the ATP/ADP ratio. An increase of the ATP/ADP ratio has been shown to decrease K^+ channel activity (Wang et al., 1990; Wang and Giebisch, 1991*b*). It is possible that inhibition of Na^+-K^+ pump turnover rate could raise the intracellular ATP concentration and reduce ADP accumulation. Such an increase of the ATP/ADP ratio would favor closure of the apical low-conductance K^+ channel. Indeed, involvement of this mechanism has been demonstrated in the rabbit proximal tubule: stimulation of the basolateral Na^+-K^+-ATPase by luminal application of organic substrates and stimulation of net Na^+ transport reduced the intracellular concentration of ATP (Tsuchiya, Wang, Giebisch, and Welling, 1992) and produced an increase of basolateral K^+ conductance which was related to the decline of ATP concentration, since intracellular ATP loading and high cell ATP levels blocked the effect of pump stimulation on basolateral K^+ conductance (Tsuchiya et al., 1992). Intracellular ATP or ADP concentrations were

not measured in the present experiments. However, it is unlikely that the same ATP/ADP mechanism played a major role in the downregulation of apical K^+ channel activity in the CCD because the effect of pump inhibition was abolished by the removal of the extracellular Ca^{2+}, a condition in which alterations of the ATP/ADP ratio should still have been effective in the downregulation of channel activity. However, the role of alterations in the ATP/ADP ratio in the upregulation of K^+ channel activity needs further exploration.

The Role of Intracellular Ca^{2+}

Three lines of evidence strongly suggest that changes in intracellular Ca^{2+} play a key role in the downregulation of K^+ channel activity after pump inhibition. First, removal of extracellular Ca^{2+} almost completely abolished the effect of reduced pump turnover on channel activity. Second, in the absence of manipulations that affect ATPase turnover, raising the intracellular Ca^{2+} concentration by ionomycin mimicked the effect of pump inhibition. The observed effect of amiloride is also consistent with a Ca^{2+}-mediated modulation of channel activity. Although amiloride is not a specific Na^+ channel blocker, it is most likely that the effect of amiloride results from blocking Na^+ channel, since other known effects of amiloride such as direct inhibition of Na^+ pump or of Na^+/H^+ exchange would enhance but not attenuate the inhibitory effect of pump inhibition on channel activity. Accordingly, it is suggested that amiloride, by blocking Na^+ channels, decreases Na^+ entry during pump inhibition. The lower intracellular concentration of Na^+ then maintains a favorable driving force for Na^+/Ca^{2+} exchange and prevents an increase in cytosolic Ca^{2+} concentration. The smaller inhibition of channel activity during pump blockade in the presence of amiloride is consistent with the maintenance of lower concentrations of Ca^{2+}.

The Role of Ca^{2+}-dependent Second Messengers

The Ca^{2+}-dependent PKC, Ca^{2+}/calmodulin–protein kinase, and Ca^{2+}-dependent phospholipase A_2 are three well-known Ca^{2+}-mediated second messengers (Klee, Crouch, and Richmam, 1980; Nishizuka, 1988; Nakamura, Nemenoff, Gronich, and Bonventre, 1991). The Ca^{2+}-dependent PKC was considered a likely candidate for a key role in the Ca^{2+}-mediated inhibition of the K^+ channel, since it had been shown that PKC-induced phosphorylation reduced channel activity and that this inhibition was dependent on the cytosolic Ca^{2+} concentration (Wang and Giebisch, 1991*a*). The evidence for an important role of PKC in the observed inhibition of channel activity is strengthened by the effects of the enzyme inhibitor, staurosporine, which significantly reduced the effect of pump inhibition on channel activity. The time delay between channel inhibition and the rise in cell Ca^{2+} (see Fig. 4) is also consistent with an indirect effect of Ca^{2+} on the involvement of mediating processes. No information is presently available concerning concentration changes of diacylglycerol (DG), which has been shown to activate PKC in the presence of Ca^{2+} and phosphatidylserine (PS). Although Ca^{2+} has no stimulatory effect on PKC without DG (Nishizuka, 1988), PKC activity becomes very sensitive to the intracellular Ca^{2+} concentration in the presence of appropriate concentrations of DG and PS (Wolf, Cuatrecasas, and Sahyoun, 1985; Nishizuka, 1988). In addition, an increase in cell Ca^{2+} could also stimulate phospho-

lipase A_2 which cleaves phospholipids to produce arachidonic acid (Nakamura et al., 1991; Cockcroft, Nielson, and Stutchfield, 1991), which is also a strong stimulator of PKC (Lester, Collin, Etcheberrigaray, and Alkon, 1991; Shinomura, Asaoka, Oka, Yoshida, and Nishizuka, 1991). We have previously shown that arachidonic acid inhibits apical low-conductance K^+ channel (Wang, Cassola, and Giebisch, 1992).

Although staurosporine is a potent PKC inhibitor (Tamaoki, Nomoto, Takahashi, Kato, Morimoto, and Tomita, 1986; Ederveen et al., 1990), we have used concentrations (200 nM) of the inhibitor that may also have blocked other Ca^{2+}-dependent kinases such as the Ca^{2+}/calmodulin-dependent protein kinase (Yanagihara, Tachikawa, Izumi, Yasugawa, Yamamoto, and Miyamoto, 1991). Calmodulin is widely distributed in mammalian cells (Klee et al., 1980) and K^+ channel modulation by Ca^{2+}/calmodulin-dependent protein kinase occurs in snail neurons (Onozuka, Furuichi, Imai, and Fukami, 1991). No information concerning the role of Ca^{2+}/calmodulin-dependent protein kinase in modulation of K^+ channels of rat CCD principal cells is presently available: accordingly we cannot exclude the possible involvement of the Ca^{2+}/calmodulin-dependent protein kinase in the downregulation of K^+ channel activity.

Although application of staurosporine markedly diminished the effect of pump inhibition on channel activity, a modest reduction of channel activity ($\sim$20%) was still apparent in the presence of this kinase inhibitor. In contrast, removal of the extracellular Ca^{2+} almost completely abolished the effect of pump inhibition on K^+ channel activity. This difference could conceivably result from the additional effect of arachidonic acid, whose production is increased by Ca^{2+} (Nakamura et al., 1991) and which has previously been shown to inhibit apical K^+ channels directly (Wang et al., 1992). However, since the major inhibitory effects of pump inhibition are abolished by staurosporine, we can safely conclude that Na^+-K^+-ATPase inhibition exerts its major inhibitory effect by activating the Ca^{2+}-dependent PKC.

Cell Model of Apical K^+ Channel Control

Fig. 11 is a model of a principal cell in the cortical collecting tubule. The proposed sequence of events includes a significant rise in cell Na^+ and, as a consequence, a diminished inwardly directed electrochemical driving force for Ca^{2+} extrusion by Na^+/Ca^{2+} exchange. It is the subsequent elevation of cell Ca^{2+} that is thought to stimulate such Ca^{2+}-dependent signal transduction pathways as PKC and phospholipase A_2, both processes known to reduce the activity of the K^+ channel in CCD principal cells (Wang and Giebisch, 1991*a*; Wang et al., 1992). In a recent study, it has been demonstrated that feedback regulation of apical Na^+ channels in CCD principal cells is also indirectly mediated by changes in cytosolic Ca^{2+} concentration when the Na^+-K^+ pump is inhibited (Frindt and Windhager, 1990; Silver et al., 1993). The model shown in Fig. 11 also contains two basolateral acid-base transporters that are also likely to be affected by the inhibition of the Na^+-K^+ pump. Na^+/H^+ exchange should decline because of the reduced driving force of Na^+ as the transmembrane Na^+ gradient across the basolateral cell membrane declines, and cell pH should fall. In addition, the activity of the electrogenic sodium-dependent HCO_3^- extrusion mechanism is likely to be compromised because basolateral Na^+-K^+ pump inhibition significantly depolarizes the basolateral membrane potential (San-

som and O'Neil, 1986; Horisberger and Giebisch, 1989). Reduced base extrusion tends to increase cell pH. The observation that cell pH remains largely unaffected by basolateral Na^+ -K^+ pump inhibition can be explained by the opposite effects on pH of the simultaneous reduction of Na/H exchange and Na^+/HCO_3^- cotransport.

The striking reduction of apical K^+ channel activity that was observed in the present experiments was closely related to significant alterations of cell Na^+ and Ca^{2+} concentrations and PKC activation. However, significant changes of net Na^+ transport and basolateral Na^+-K^+ ATPase turnover may not always involve major

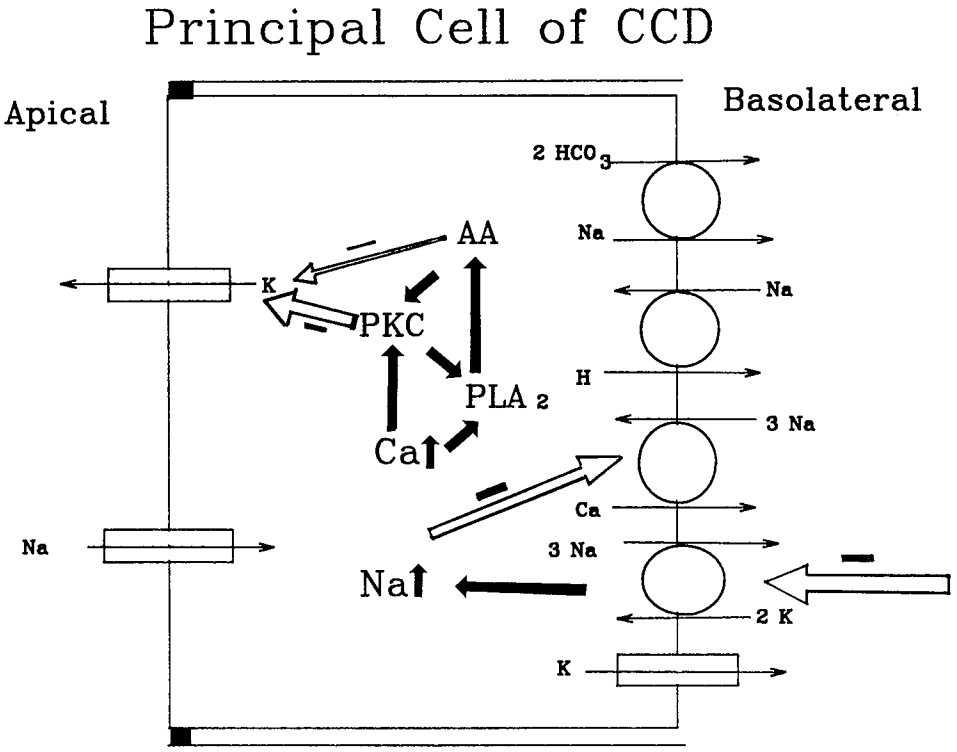

FIGURE 11. Cell scheme of coupling mechanism linking inhibition of basolateral Na^+-K^+ pump activity to inactivation of apical K^+ channel activity. Open arrows indicate inhibition or downregulation, whereas solid arrows indicate upregulation or increase of electrolyte concentrations. *AA*, arachidonic acid; *PKC*, Ca^{2+}-dependent protein kinase C; *PLA_2*, phospholipase A_2.

derangements of cell Na^+ and Ca^{2+}, particularly when active basolateral pump stimulation is appropriately matched by increased Na^+ permeability. Increased luminal Na^+ delivery to the cortical collecting tubule or aldosterone administration (Hropot, Fowler, Karlmark, and Giebisch, 1985; Sansom and O'Neil, 1985; Field and Giebisch, 1990; Stanton and Giebisch, 1992; Wright and Giebisch, 1992), both conditions in which tubular reabsorption of sodium and secretion of potassium are stimulated, are relevant examples. Under these conditions it is possible that additional mechanisms are activated and account for modulation of K^+ channel activity in

concert with changes in Na^+-K^+-ATPase turnover. Since the apical K^+ channel is not sensitive to membrane voltage, it is virtually certain that changes in apical membrane potential are not involved. It is also of interest to consider the role of the ATP/ADP ratio in coordinating K^+ channel activity with Na^+-K^+ pump turnover rate. It has been reported in studies on cortical thick ascending limbs that cell ATP levels are affected by alterations of sodium transport such that decreased apical Na^+ entry maintains cell ATP levels at higher levels than when Na^+ is permitted to enter the cell (Uchida and Endou, 1988). Additional experiments will be necessary to explore the possibility that changes in cell ATP are involved in the transport-related modulation of the K^+ channel under those conditions in which no major changes in cell Na^+ and Ca^{2+} occur.

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