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Time- and Dose-Dependent Effects of Protein Kinase C on Proximal Bicarbonate Transport

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Summary. Activation of protein kinase C has been shown to cause both stimulation and inhibition of transport processes in the brush-border membrane and renal tubule. This study was designed to examine the dose-response nature and time-dependent effect of 4 β-phorbol-12-myristate-13-acetate (PMA) on the rates of bicarbonate absorption (J_{HCO_3}) and fluid absorption (J_v) in the proximal convoluted tubule (PCT) of rat kidney. Bicarbonate flux was determined by total CO_2 changes between the collected fluid and the original perfusate as analyzed by microcalorimetry. Luminal perfusion of PMA ($10^{-10} \sim 10^{-5}$ M) within 10 min caused a significant increase of J_{HCO_3} and J_v. A peaked curve of the dose response was observed with maximal effect at 10^{-8} M PMA on both bicarbonate and fluid reabsorption, which could be blocked completely by amiloride (10^{-3} M) and EIPA (10^{-5} M). On the other hand, with an increase of perfusion time beyond 15 min, PMA (10^{-8} and 10^{-6} M) could inhibit J_{HCO_3} and J_v. Amiloride (10^{-3} M) or EIPA (10^{-5} M) significantly inhibits J_{HCO_3} and J_v, while there is no additive effect of PMA and amiloride or EIPA on PCT transport. An inactive phorbol-ester, 4α-phorbol, that does not activate protein kinase C, had no effects on J_{HCO_3} and J_v. Capillary perfusion of PMA (10^{-8} M) significantly stimulate both J_{HCO_3} and J_v; however, PMA did not affect glucose transport from either the luminal side or basolateral side of the PCT. These results indicate that activation of endogenous protein kinase C by PMA could either stimulate or inhibit both bicarbonate and fluid reabsorption in the PCT dependent on time and dose, and these effects are through the modulation of Na^+/H^+ exchange mechanism.

Key Words protein kinase C · bicarbonate transport · fluid absorption · sodium/hydrogen exchange · time- and dose-dependent effect of PMA · renal proximal tubule

Introduction

Protein kinase C, a Ca^{2+}-sensitive phospholipid-dependent protein phosphorylase, appears to play an important role in the modulation of signal transduction [19], hormone regulation [11, 13] and membrane transport processes [1, 2, 5, 8, 12] in many cell systems. It was also demonstrated that protein kinase C (PKC) may have an important influence on the development of electrolyte transport in the kidney proximal tubule [15]. In previous studies, evidence has been advanced that protein kinase C is present in the brush border membrane and basolateral membrane of the renal proximal tubular cell [14, 16, 25].

In brush-border membrane vesicles from rabbit kidney, activation of PKC results in stimulation of the Na^+/H^+ exchanger [24, 25]. In contrast, phorbol esters (activators of PKC) have been shown to inhibit volume reabsorption in the rabbit proximal tubule [3]. Of particular interest is the observation in brush-border membranes that activation of PKC results in stimulation [17, 25] or inhibition of sodium transport [3]. Some investigators indicated that a stimulation of Na^+/H^+ exchange should have resulted in an increase in bicarbonate transport. The reason for the apparent discrepancy in results is unclear at present [3]. Therefore, it is of interest to investigate if PKC regulates bicarbonate transport, whether PKC stimulates or inhibits Na^+/H^+ exchange in the renal proximal tubule in vivo, and to study the relationship of stimulatory and inhibitory effects of PKC on sodium transport processes to find out the reason for the discrepancy in the results of the previously studies.

This study was designed to examine the effect of phorbol ester (PMA) on proximal tubular bicarbonate and fluid transport in rat kidney with particular attention to their dose- and time-dependent effects on the Na^+/H^+ exchange.

Materials and Methods

Micropuncture experiments were conducted on 76 male Sprague-Dawley rats weighing 261.4 ± 4.5 g. All animals were allowed free access to regular Purina chow and tap water until the experiment. Rats were anesthetized by intraperitoneal injection of 100 mg/kg body weight of 5-ethyl-5-(L-methylpropyl)-2-thiobarbi-

turic acid (Inactin) and were placed on a thermostatically controlled surgical table to maintain body temperature at 37°C. A tracheostomy was performed. The left jugular vein was cannulated with two catheters, one for infusion of saline at a rate of 1.5 ml/hr, and the other to administer an additional 1 ml of the saline during the surgical preparation. The left carotid artery was cannulated with a PE-50 polyethylene catheter. Mean carotid artery pressure was recorded during the whole experimental period in order to confirm that the blood pressure remained above 90 mmHg.

The left kidney was exposed laterally by a left flank incision and then immobilized in a double kidney cup (W. Hampel, Frankfurt, FRG). The kidney was submerged in light mineral oil throughout the experiment to maintain the kidney temperature at 37°C. The left ureter was catheterized with a short piece of PE-10 tubing to allow free flow of urine. The kidney was observed under a stereomicroscope and illuminated by a fiber-optic light source.

The technique of simultaneous microperfusion of proximal convoluted tubule and peritubular capillaries has been described previously [6, 7]. First, loops of proximal convoluted tubules on the kidney surface were selected by injection of a tiny drop of colored paraffin oil, and then perfused at a rate of 16 nl/min with a microperfusion pump (Type III with electronic feedback speed control, W. Hampel, Max-Planck-Institute of Biophysics, Frankfurt, FRG); the oil block was injected up to the perfusion site. The perfusion solution usually contained 0.1% FD&C green dye, which allowed identification of the perfused loops in proximal tubules. The collection pipette (tip o.d. 10–11 μm) was filled with stained (Sudan Black) heavy mineral oil to collect perfusate. Time controlled total collections of perfusate were made downstream with another micropipette with distal oil block. After each collection the pipette was withdrawn from the tubule into the oil covering the surface of the kidney, and a small amount of oil was aspirated into the tip of the collection pipette in order to prevent evaporation of the sample.

At the end of the experiment, perfused tubules were filled with high viscosity microphil (Canton Bio-Medical Products, Boulder, CO). The kidney was excised and stored overnight in deionized water at 4°C. On the day after the experiment, the kidney was partially digested in 25% NaOH for 25 min and the latex casts of tubules were dissected. Using a drawing device for a Wild stereomicroscope, both the casts and a known distance from an object micrometer were drawn and the length of the perfused tubule was measured by comparison. The perfusion solutions were as follows (in mM) sodium chloride 110, sodium bicarbonate 25, potassium chloride 1, sodium acetate 5, glucose 5, alanine 5, dibasic sodium phosphate 2.5, monobasic sodium phosphate 0.5. Solutions were bubbled at room temperature with a 5% CO_2, 95% O_2 gas mixture before use. Subsequentiy, the pH of these solutions was adjusted to 7.4 with a small amount of NaOH or HCl. Low sodium ^{3}H-inulin (20 μCi/ml) was also added to the tubular perfusate. The calibration of the perfusion pump used for tubular perfusion was checked before and after each experiment. The bicarbonate concentration in the perfusate and collected fluid was determined using a microcalorimetric (picapnotherm) method as described previously [7]. Briefly, collected samples were stored under oil and 10-nl aliquots were compared with 5, 10, 15, and 25 mM sodium carbonate standards. The calibration curve was linear and a correlation coefficient greater than 0.99 was determined in all cases.

The fluid volume of the original and collected samples was measured in a constant-bore glass capillary. Samples were analyzed for radioactivity using a liquid scintillation counter.

The rate of net fluid reabsorption (J_v) is calculated according to the following equation:

$$J_v = V_o - V_L \tag{1}$$

where V_L = measured rate of fluid collection at length L, $V_o = V_L[IN_L/IN_o]$, and IN_L/IN_o = ratio of radioactive inulin of collected and perfused fluid. The net flux of a compound J_S, is given by the equation:

$$J_S = V_o[S]_o - V_L[S]_L \tag{2}$$

where $[S]_o$ = concentration of compound in the original perfusate and $[S]_L$ = concentration of compound in the collected fluid; these quantities were expressed per mm of tubular length.

All data are presented as mean ± SE. Donnett's test was used to compare mean values between groups of experiments. Differences between groups are reported as significant at $P < 0.05$.

Materials

[^{3}H]-inulin, [^{14}C]-inulin and [^{3}H]-glucose were purchased from New England Nuclear (Boston, MA), PMA, 4α-phorbol 12,13-didecanoate and amiloride were purchased from Sigma Chemical (St. Louis, MO). EIPA (ethylisopropyl-amiloride) was obtained from Yale University.

Results

Microperfusion experiments in vivo were conducted in 306 proximal convoluted tubules. The average of the perfused tubular length was 1.79 ± 0.07 mm. Controls were perfused with normal Ringer's solution containing 25 mM $NaHCO_3$ and 1 mM $CaCl_2$. The arterial blood gas values were: pH = 7.36 ± 0.02; pCO_2 = 44.1 ± 0.32 mmHg; HCO_3^- = 24.45 ± 0.67 mM. There were no significant changes between the experimental group and controls.

Bicarbonate reabsorption (J_{HCO3}) was 140.3 ± 3.83 pEq/min · mm (n = 17) and fluid reabsorption (J_v) was 2.51 ± 0.15 nl/min · mm (n = 17) in the control group with 10 min PCT perfusion. There were no significant changes of J_{HCO3} and J_v, when the perfusion time was extended to 25 or 35 min.

Dose-Dependent Effect of PMA

The dose-response relationship between PMA and bicarbonate and fluid reabsorption, was tested by perfusing proximal convoluted tubule (PCT) for less than 10 min with six different doses of PMA from 10^{-10} to 10^{-5} M. Data from these experiments are given in Table 1. The dose-response curves are shown in Fig. 1.

After PMA treatment of 10^{-9} to 10^{-5} M, the

Table 1. Summary data of the effects of PMA and amiloride on fluid and bicarbonate reabsorption in the proximal convoluted tubule of rat kidney (perfusion time was less than 10 min)

	n	V_v (nl/min)	$[HCO_3]_o$ (mEq/liter)	$[HCO_3]_L$ (mEq/liter)	L (mm)	J_v (nl/min · mm)	J_{HCO3} (pEq/min · mm)
Control	17	16.24 ± 0.11	25.3 ± 0.52	12.8 ± 0.65	1.88 ± 0.08	2.51 ± 0.15	140.3 ± 3.83
PMA 10^{-10} M	23	16.63 ± 0.27	26.6 ± 0.31	15.4 ± 0.83	1.80 ± 0.10	3.02 ± 0.18	153.2 ± 7.69
PMA 10^{-9} M	12	17.09 ± 0.53	25.1 ± 0.33	15.2 ± 0.87	1.56 ± 0.15	3.55 ± 0.27[a]	165.3 ± 6.94[a]
PMA 10^{-8} M	14	16.49 ± 0.16	25.9 ± 0.21	10.6 ± 1.09	1.74 ± 0.10	4.04 ± 0.32[a]	187.0 ± 7.84[a]
PMA 10^{-7} M	12	17.41 ± 0.13	25.2 ± 0.53	17.1 ± 0.77	1.17 ± 0.08	3.57 ± 0.25[a]	170.9 ± 10.58[a]
PMA 10^{-6} M	16	16.57 ± 0.38	25.7 ± 0.35	16.4 ± 0.54	1.57 ± 0.13	3.30 ± 0.24[a]	164.7 ± 9.66[a]
PMA 10^{-5} M	12	16.04 ± 0.01	24.5 ± 0.07	14.2 ± 1.32	1.28 ± 0.13	3.12 ± 0.26[a]	165.0 ± 10.56[a]
4α-phorbol 10^{-6} M	6	16.69 ± 0.49	25.5 ± 0.0	15.7 ± 1.2	1.71 ± 0.26	2.30 ± 0.31	135.8 ± 11.20
AMIL 10^{-3} M	11	15.85 ± 0.24	24.5 ± 0.25	16.9 ± 0.79	1.92 ± 0.21	1.78 ± 0.19[a]	97.2 ± 6.92[a]
PMA 10^{-8} M + AMIL 10^{-3} M	13	16.04 ± 0.01	24.2 ± 0.15	16.2 ± 0.78	2.18 ± 0.17	2.16 ± 0.26[a]	96.5 ± 8.12[a]
AMIL 10^{-4} M	17	15.53 ± 0.06	25.3 ± 0.11	12.3 ± 0.98	2.16 ± 0.17	2.22 ± 0.20	117.9 ± 6.87
PMA 10^{-8} M + AMIL 10^{-4} M	15	16.57 ± 0.12	25.0 ± 0.14	11.3 ± 0.94	1.86 ± 0.13	2.78 ± 0.11	152.7 ± 4.17
EIPA 10^{-5} M	9	16.22 ± 0.07	24.7 ± 0.15	12.8 ± 1.46	2.38 ± 0.33	1.38 ± 0.23[a]	91.8 ± 1.84[a]
EIPA 10^{-5} M + PMA 10^{-8} M	8	15.96 ± 0.04	25.7 ± 0.17	16.1 ± 2.15	1.99 ± 0.23	1.58 ± 0.31[a]	87.7 ± 4.43[a]

n: number of perfused tubule; $[HCO_3]_o$: bicarbonate concentration in the original perfusate; $[HCO_3]_L$: bicarbonate concentration in the collected fluid; PMA: 4-β-phorbol-12-myristate-13-acetate; AMIL: amiloride; EIPA: ethylisopropylamiloride.
[a] Significantly different $P < 0.05$.

rates of both bicarbonate and fluid reabsorption were significantly higher than the control group. The maximal stimulation dose was 10^{-8} M under these conditions. J_v was increased 61% from 2.51 ± 0.15 nl/min · mm to 4.04 ± 0.32 nl/min · mm ($P < 0.001$; $n = 14$), J_{HCO3} was increased 33% from 140.3 ± 3.83 pEq/min · mm to 187.0 ± 7.84 pEq/min · mm ($P < 0.001$; $n = 14$). In another series of experiments, J_v was increased 41% ($P < 0.02$; $n = 12$) and 42% ($P < 0.02$; $n = 12$), J_{HCO3} was increased 18% ($P < 0.02$; $n = 12$) and 21% ($P < 0.02$; $n = 12$) in the 10^{-9} and 10^{-7} M treatment groups of PMA, respectively.

The significant increase of J_v and J_{HCO3} was also observed at 10^{-6} and 10^{-5} M PMA. However, these stimulatory effects on J_v and J_{HCO3} are lower than the maximal effects seen at 10^{-8} M. There were no significant changes of J_v and J_{HCO3} at 10^{-10} M PMA.

As shown in Fig. 1, PMA (10^{-9} to 10^{-5} M) significantly enhanced fluid and bicarbonate reabsorption by proximal convoluted tubules. The peaked curves of dose-response relationship of PMA were observed with maximal stimulation of J_{HCO3} and J_v at 10^{-8} M.

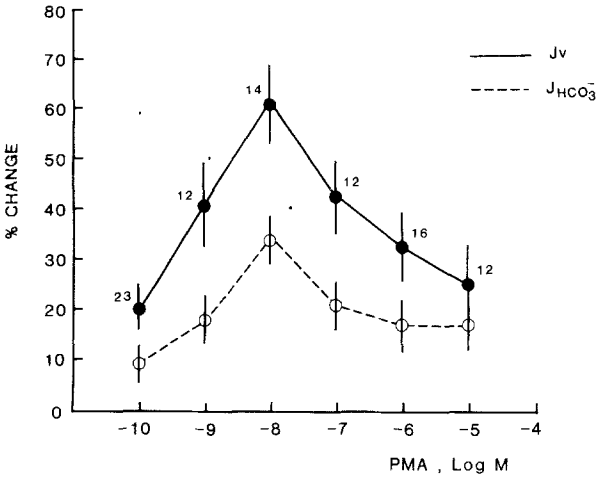

Fig. 1. Dose-response effects of PMA on bicarbonate (J_{HCO3}) and fluid (J_v) reabsorption in proximal convoluted tubules (PCT). PMA was added to the luminal perfusate at concentrations of 10^{-10} to 10^{-5} M. Perfusion time was less than 10 min. Numbers represented in this figure are the numbers of the perfused tubule. Significant increases in both J_v and J_{HCO3} were observed at concentrations from 10^{-9} to 10^{-5} M ($P < 0.05$)

Time-Dependent Effect of PMA on Bicarbonate and Fluid Reabsorption in PCT

Time-dependent effects of PMA on fluid and bicarbonate reabsorption were studied by perfusing proximal convoluted tubules with two different doses of PMA, 10^{-8} and 10^{-6} M, in three different time periods: 0–10, 15–25 and 25–35 min.

In this experiment, PCT were continuously perfused in the presence of PMA in the luminal perfusate at concentrations of 10^{-8} and 10^{-6} M. Three collections were made in each proximal tubule at the different perfusion periods, and each collection was not longer than 10 min.

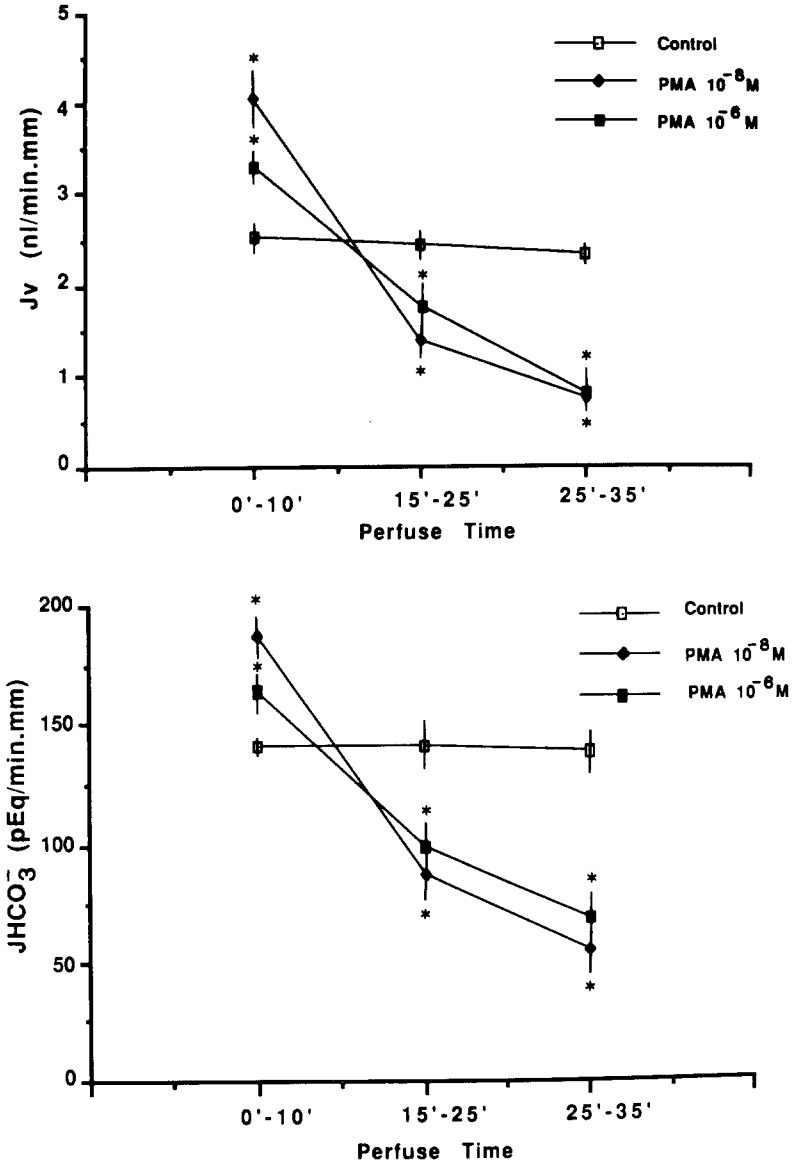

Fig. 2. Time-dependent effects of PMA on bicarbonate (J_{HCO3}) and fluid (J_v) reabsorption in proximal convoluted tubules (PCT). PMA was added to the luminal perfusate at concentrations of 10^{-8} and 10^{-6} M. Asterisks indicate significantly different from control values ($P < 0.05$)

In the first period (0–10 min), the collection of the tubular fluid was taken immediately followed by PMA administration and finished before 10 min of the perfusion time. During the second period (15–25 min), the collection was started at 15 min of PMA adminstration and finished before 25 min of perfusion time. The third collection was started at 25 min of perfusion and finished before 35 min of perfusion time. Control groups were perfused under similar conditions without PMA. Inactive phorbol ester 4α-phorbol 12,13 didecanoate was also tested in this study. Data from these experiments are shown in Fig. 2. PMA (10^{-8} and 10^{-6} M, with 10 min perfusion) immediately increased J_v by 61 and 32% ($P < 0.02$) and increased J_{HCO3} by 33 and 17% ($P < 0.05$), respectively.

Of particular interest is that the stimulatory effects of J_v and J_{HCO3} were reversed by prolonged perfusion of PMA. In the second period (15–25 min) J_v was decreased 45% ($P < 0.01$; $n = 8$) and 30% ($P < 0.05$; $n = 7$); J_{HCO3} was decreased 37.9% ($P < 0.005$; $n = 8$) and 29% ($P < 0.05$; $n = 7$) in 10^{-8} and 10^{-6} M PMA treatment groups, respectively.

Increased inhibition of J_v and J_{HCO3} was observed during the (25–35 min) perfusion period. J_v was decreased 70 and 68% ($P < 0.001$); J_{HCO3} was decreased 60 and 51% ($P < 0.001$) in 10^{-8} and 10^{-6} M PMA treatment groups, respectively.

Figure 2 also indicates that both stimulatory and inhibitory effects of 10^{-8} M PMA were stronger than that seen with 10^{-6} M PMA. However, this difference was not significant. In the control groups

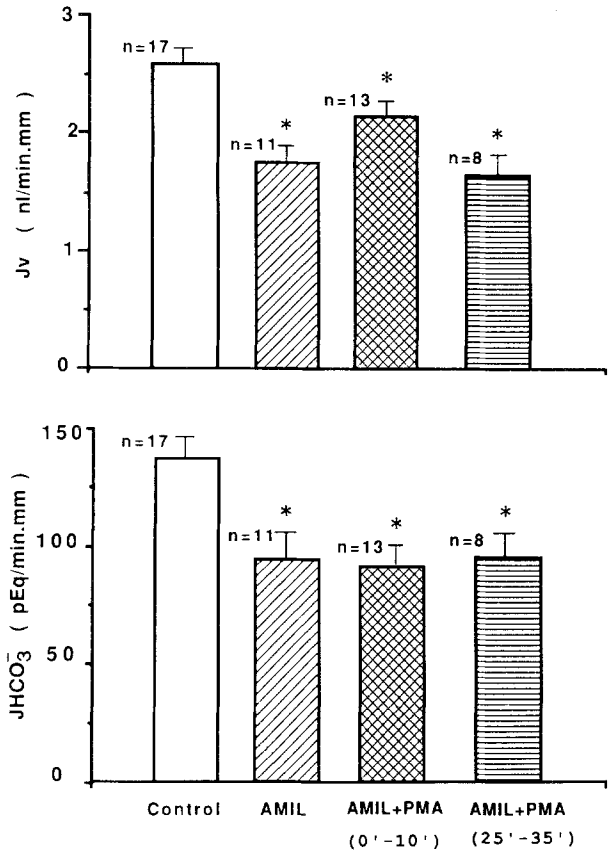

Fig. 3. Effects of amiloride (AMIL) on PMA-induced stimulation and inhibition of bicarbonate (J_{HCO3}) and fluid (J_v) reabsorption in proximal convoluted tubules (PCT). Four different bars shown in this figure are control, amiloride, amiloride plus PMA with 10 min perfusion and amiloride plus PMA with 25–35 min perfusion. Both PMA and amiloride were added to the luminal perfusate at concentrations of 10^{-8} and 10^{-3} M, respectively. n = number of perfused tubules. Asterisks indicate significantly different from control value ($P < 0.05$)

(without PMA), there were no significant changes of J_v and J_{HCO3} when groups from prolonged perfusion times were compared to the 0–10 min period group. Additionally, J_v and J_{HCO3} did not changed by perfusing 4α-phorbol ester with (10^{-6} M) three different perfusion times.

Effects of Amiloride and Ethylisopropylamiloride on PMA-Induced Stimulation and Inhibition of Bicarbonate and Fluid Reabsorption in the PCT

Since the activation of protein kinase C by PMA can stimulate or inhibit both bicarbonate and fluid reabsorption, it is of interest to investigate whether the Na^+/H^+ exchange is the target mechanism for PKC actions. Thus, we used amiloride as an inhibitor of the sodium and hydrogen exchange mechanism. First, we tested if amiloride could block the PMA-induced stimulatory effect of J_{HCO3} and J_v. As shown in Fig. 3 and Table 1, 10^{-3} M amiloride significantly decreased fluid and bicarbonate reabsorption when PMA (10^{-8} M) and amiloride (10^{-3} M) were added together to the luminal perfusate for 10 min. Under these conditions, the rate of bicarbonate and fluid reabsorption were quite similar to that of treatment with 10^{-3} M amiloride alone. These experiments show that the stimulatory effect of PMA on J_{HCO3} and J_v was completely blocked by amiloride.

Since long-term perfusion of PMA and amiloride could inhibit both fluid and bicarbonate reabsorption, further experiments were performed to investigate if they have additive effect. PMA (10^{-8} M) and amiloride (10^{-3} M) were added simultaneously to the luminal perfusate and collected during the 25–35 min period. As shown in Fig. 3 there was no further inhibition of J_{HCO3} and J_v, when PMA and amiloride were added together as compared to treatments with PMA alone or amiloride alone. Since there was no additive effect on either J_{HCO3} or J_v when PMA and amiloride were perfused together, the data suggests that PMA and amiloride act by a similar mechanism that inhibits the Na^+/H^+ exchange. It should be noted, however, that the dose of amiloride used in this study might have some nonspecific effects other than inhibiting Na-H exchanger, thus a more specific Na-H exchanger inhibitor, ethylisopropylamiloride (EIPA) was used in additional microperfusion experiments. Data from these experiments are given in Table 1 and Fig. 4. It can be seen that a lower dose (10^{-5} M) of EIPA is required to inhibit J_v and J_{HCO3} to a similar extent as amiloride (10^{-3} M). EIPA (10^{-5} M) also completely blocked the effect of PMA (10^{-8} M).

Effect of PMA on Glucose Transport in PCT

In order to examine whether PMA can affect another Na-dependent transport process, a series of experiments was performed to study the luminal effect of PMA on glucose transport in PCT. In these experiments, [^{3}H]-glucose and nonlabeled glucose were added in the luminal perfusate at the final concentration of 5.2 mM, and [^{14}C]-inulin was added as a volume market. The results are presented in Table 2. PMA (10^{-8} M) had no effect on glucose transport despite stimulating J_v with 10-min perfusion and inhibiting J_v with 15–25 min perfusion.

Table 2. The effects of luminal perfusion of PMA on fluid and glucose reabsorption in the proximal convoluted tubule

	n	V_o (nl/min)	$[Glu]_o$ (mM)	$[Glu]_L$ (mM)	L (mm)	J_v (nl/min · mm)	J_{gl} (pM/min · mm)
Control	11	16.08 ± 0.04	5.2 ± 0.01	2.2 ± 0.47	1.91 ± 0.16	2.69 ± 0.19	32.54 ± 2.19
PMA 10^{-8} M[a]	10	16.31 ± 0.09	5.2 ± 0.01	2.7 ± 0.41	1.74 ± 0.10	4.10 ± 0.29[c]	33.98 ± 2.68
PMA 10^{-8} M[b]	8	16.31 ± 0.10	5.2 ± 0.01	3.7 ± 0.25	1.41 ± 0.18	1.53 ± 0.23[d]	29.74 ± 2.28

n: number of perfused tubule; $[Glu]_o$: glucose concentration in the original perfusate; $[Glu]_L$: glucose concentration in the collected fluid. J_{gl}: rate of glucose absorption.
[a] Luminal perfusion of PMA, perfusion time was less than 10 min.
[b] Luminal perfusion of PMA, perfusion time was 15–25 min.
[c] Significantly different $P < 0.001$.
[d] Significantly different $P < 0.005$.

Effect of PMA from the Basolateral Side of PCT

To examine the basolateral effect of PMA on PCT transport, combined capillary and luminal perfusion experiments were performed. The results were shown in Table 3. It was demonstrated that 10 min perfusion of 10^{-8} M PMA in the capillary perfusate significantly increased J_v and J_{HCO3} to a similar extent as luminal perfusion. It also showed that capillary perfusion PMA had no effect on glucose transport. Due to technical difficulty of capillary microperfusion, the effect of longer perfusion of PMA was not examined.

Discussion

It is well known that tumor-promoter phorbol esters such as PMA, have a structure similar to diacylglycerol and activate protein kinase C directly [5, 10, 26]. Because phorbol esters have been widely used to investigate the action of protein kinase C, we used PMA to activate the endogenous PKC from the apical and basolateral side of the proximal convoluted tubule to investigate the physiological significance of PKC on bicarbonate and fluid transport in the renal proximal convoluted tubule.

An important way to study the characteristics of an agent is to test the effect of several different concentrations of the agent, especially changing the concentrations as the log M. For this reason, six different concentrations of PMA from 10^{-10} to 10^{-5} M were used to study the effects of PMA on bicarbonate and fluid reabsorption in PCT. Factors that could influence tubular transport were controlled very carefully. These included perfusion rate, sodium bicarbonate concentrations of the perfusate and perfusion time.

Data from this study, as shown in Fig. 1 and Table 1, indicated that PMA (10^{-9} to 10^{-5} M) significantly stimulated both bicarbonate and fluid reabsorption in PCT. Figure 3 and Table 1 show that the stimulatory effects of PMA on J_{HCO3} and J_v were completely blocked by amiloride, indicating the stimulatory effect of PMA on bicarbonate and fluid

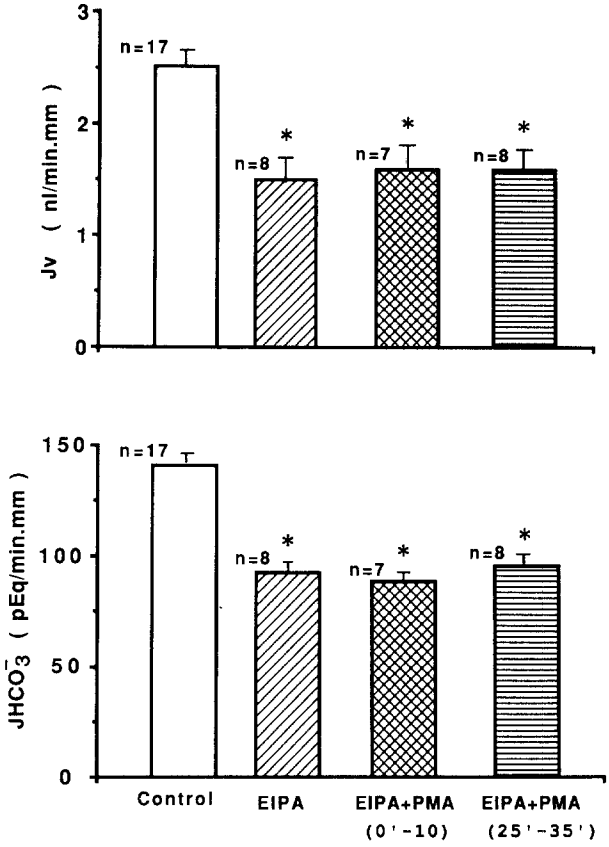

Fig. 4. Effects of ethylisopropylamiloride (EIPA) on PMA-induced stimulation and inhibition of bicarbonate (J_{HCO3}) and fluid (J_v) reabsorption in proximal convoluted tubules (PCT). Four different bars shown in this figure are control, EIPA, EIPA plus PMA with 10 min perfusion and EIPA plus PMA with 25–35 min perfusion. Both PMA and EIPA were added to the luminal perfusate at concentrations of 10^{-8} and 10^{-5} M, respectively. n = number of perfused tubules. Asterisks indicate significantly different from control value ($P < 0.05$)

Table 3. Effects of capillary perfusion of PMA on fluid, bicarbonate and glucose transport in the proximal convoluted tubule of rat kidney

	n	V_o (nl/min)	$[HCO_3]_o$ (mEq/liter)	$[HCO_3]_L$ (mEq/liter)	$[Glu]_o$ (mM)	$[Glu]_L$ (mM)	L (mm)	J_v (nl/min · mm)	J_{HCO_3} (pEq/min · mm)	J_{gl} (PM/min · mm)
Control	10	19.8 ± 0.08	25.4 ± 0.1	14.3 ± 0.71	5.2 ± 0.01	2.1 ± 0.42	2.02 ± 0.17	2.54 ± 0.15	143.8 ± 8.3	36.33 ± 2.54
PMA 10^{-8} M	12	19.8 ± 0.08	25.7 ± 0.1	13.5 ± 1.58	5.2 ± 0.01	2.8 ± 0.35	1.72 ± 0.09	4.55 ± 0.32[a]	201.6 ± 8.4[a]	39.65 ± 1.30

n: number of perfused tubule; $[HCO_3]_o$: bicarbonate concentration in the original perfusate; $[HCO_3]_L$: bicarbonate concentration in the collected fluid; $[Glu]_o$: glucose concentration in the original perfusate; $[Glu]_L$: glucose concentration in the collected fluid.
[a] Significantly different $P < 0.001$. J_{gl}: rate of glucose absorption.

reabsorption is amiloride sensitive. These results confirm and extend the results from previous studies in which amiloride inhibited phorbol ester-stimulated Na^+/H^+ exchanger in the brush border membrane [24, 25].

Protein kinase C has been reported to be able to stimulate Na^+/H^+ exchanger in many different cell systems [4, 17, 23]. Conversely, phorbol esters have been shown to inhibit an amiloride-sensitive Na^+/H^+ exchanger in the luminal membrane of the proximal colon [1]. PMA and dioctanoylglycerol have been shown to inhibit volume and bicarbonate absorption in the renal proximal convoluted tubule [3]. However, we have noticed that most of these studies using different cell systems were performed in vitro and the incubation times were significantly different.

A short incubation (10 sec to 5 min) was used in those studies where PKC stimulated sodium transport [4, 17, 25], whereas a longer incubation time (30 min) was used in those studies where PKC inhibited sodium transport [1, 3]. Therefore, it is of interest to investigate if PKC has a time-dependent, biphasic effect.

Using microperfusion technique, 5–8 min was usually needed to collect enough perfusate for analysis. Three different perfusion periods were selected (0–10, 15–25 and 25–35 min) for this study. The time course of the control group was studied in this experiment in order to observe changes in J_v and J_{HCO_3} in the different perfusion periods. Changes induced by prolonged perfusion times were also noted. An inactive phorbol ester (4α-phorbol [3, 22, 25] was also studied in this experiment using the three different perfusion periods, in order to exclude effects caused by mechanisms of the phorbol esters which were not through activation of PKC.

The data from these control experiments indicated that there were no significant changes of J_v and J_{HCO_3} when perfusion time was prolonged. Similar values of J_v and J_{HCO_3} were obtained in the three different perfusion time periods. As shown in Table 1, 4α-phorbol also did not induce significant effects on J_v and J_{HCO_3} in any of the three different perfusion time periods.

However, we have observed that PMA did induce time-dependent, biphasic effects on bicarbonate and fluid absorption. Figure 2 indicates that PMA at 10^{-8} and 10^{-6} M, significantly stimulates J_v and J_{HCO_3} in the short time perfusion period (0–10 min) while they significantly inhibit both J_v and J_{HCO_3} in the perfusion time beyond 15 min.

These results are supported by data from previous studies where PKC stimulates the Na^+/H^+ exchanger in the renal brush border membrane [24, 25] and PKC inhibits volume and bicarbonate transport in the proximal tubule [3]. Since PMA has been shown to inhibit both bicarbonate and fluid reabsorption in our (25–35 min perfusion) experiment, it is possible that Na^+/H^+ exchange is involved in this mechanism. Therefore, it is of interest to investigate if the inhibitory effects of PMA on J_v and J_{HCO_3} are similar to the mechanism of amiloride. Figure 3 and Table 1 indicate that 10^{-3} M amiloride significantly inhibits J_v and J_{HCO_3}; however, there was no additive effect of amiloride and PMA (10^{-8} M) on J_v and J_{HCO_3} in the 25 to 35 min perfusion period. According to these results, it is possible that PKC also could inhibit Na^+/H^+ exchange in the renal brush border membrane. Since it was demonstrated that amiloride could also inhibit phorbol ester-stimulated and PKC-mediated phosphorylation in human leukemic cell line [4], the more specific amiloride analog, ethylisopropylamiloride was used for further study. As seen from Table 1 and Fig. 4, 10^{-5} M ethylisopropylamiloride had an effect similar to that of 10^{-3} M amiloride, namely, inhibition of J_v and J_{HCO_3}. It also blocked the effect of PMA, thus, suggesting that PMA has a specific effect via acting on Na-H exchanger. This view was further confirmed by the observation that PMA had no effect on glucose transport, another Na-dependent transport process in PCT (Tables 2 and 3). PMA can also exert its effect on J_v and J_{HCO_3} from the basolateral side (Table 3). These results suggest that PMA can enter from both the apical and basolateral membrane and bind to PKC in the cytosol [18]. Subse-

quently, PKC is rapidly translocated from the cytosol to the apical membrane, resulting in modulation of Na-H exchanger.

It is well known that PKC plays an important role in regulation of signal transduction processes [18] and appears to be intimately related to positive signal transduction. The down-regulation mechanism through feedback control by PKC has also been proposed for intracellular processes, such as smooth muscle contraction [20]. Negative feedback control by PKC, however, has not been reported in membrane transport processes. In our study, evidence has been revealed that PMA inhibits the bicarbonate and fluid transport processes in renal PCT, presumably by down-regulation mechanism. The data from our experiments have shown that PMA initially stimulates bicarbonate and fluid transport followed by the down-regulated process where bicarbonate and fluid transport appear to be inhibited.

The dose-response curve of PMA also supports a down-regulation mechanism. This relationship results in a biphasic curve in our study (Fig. 1). It is interesting to observe that after the maximal stimulatory effect on J_v and J_{HCO3} by 10^{-8} M PMA, followed by down-regulation, the stimulation effect on both J_v and J_{HCO3} were getting lower and lower when PMA concentration increased. It has been proposed that the binding of phorbol esters to their receptors, presumably PKC, resulted in a decrease of the binding capacity of the receptors on subsequent exposure to phorbol esters. This down-regulation phenomenon has been observed in several cells [9, 21]. Treatment of intact 3T3 cells with a phorbol ester, phorbol 12,13 dibutyrate, causes a dose and time-dependent decrease in the PKC activity measured in cell free and detergent-solubilized extracts [21]. The biochemical mechanism of down-regulation of the binding of phorbol esters to their receptors has been studied in a cultured cell line of fetal rat skin keratinocytes [9]. This study indicated that when these cells were treated with a 12-0-tetra-decanylphorbol-13-acetate (TPA), PKC was rapidly translocated from the cytosol to the membranes and then both PKC activity and binding of TPA decreased rapidly. It was suggested that nonlysosomal proteolytic degradation of PKC is a cause of down-regulation of phorbol ester receptors. Alternately, it is possible that PKC regulates bicarbonate and fluid transport processes via increased activation of the Na^+/H^+ exchanger and down-regulation of the Na^+/H^+ exchange mechanism. However, the mechanism by which PKC regulates the Na^+/H^+ exchanger is still under investigation.

In summary, the important findings in this study are that, first, PMA but not 4α-phorbol significantly stimulates both bicarbonate and fluid reabsorption in the PCT. Second, the dose-response curves of PMA on J_v and J_{HCO3} are biphasic with a maximal stimulation dose of 10^{-8} M. Third, PMA significantly inhibits both bicarbonate and fluid reabsorption in the longer perfusion period beyond 15 min. Finally, both stimulation and inhibition effects of PMA on J_v and J_{HCO3} are amiloride and EIPA sensitive, suggesting that Na-H exchanger is subject to modulation of PKC.

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