

Role of Protein Kinase C in Parathyroid Hormone Stimulation of Renal 1,25-Dihydroxyvitamin D₃ Secretion

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Abstract

PTH is a major regulator of renal proximal tubule 1,25(OH)₂D₃ biosynthesis. However, the intracellular pathways involved in PTH activation of the mitochondrial 25-hydroxyvitamin D₃-1 alpha-hydroxylase (1-OHase) remain unknown. PTH can activate both the adenylate cyclase/protein kinase A (PKA) and the plasma membrane phospholipase C/protein kinase C (PKC) pathways. The present study was undertaken to determine whether PKC may mediate PTH activation of renal 25-hydroxyvitamin D₃-1 alpha-hydroxylase activity. Rat PTH 1-34 fragment in vitro translocated PKC activity from cytosolic to soluble membrane fraction from freshly prepared rat proximal tubules. Physiologic concentrations (10⁻¹¹–10⁻¹⁰ M) of rat PTH 1-34 fragment increased PKC translocation three- to fourfold while PKA activity ratio increased at PTH 10⁻⁷ M. PTH stimulation of PKC and PKA was reduced in the presence of staurosporine (10 nM) by 41 and 29%, respectively. Sangivamycin (10 and 50 μM) also reduced PTH-stimulated PKC translocation, but did not alter PKA activity ratio.

In vitro perfusion of renal proximal tubules with PTH (10⁻¹¹ M) increased 1,25(OH)₂D₃ steady-state secretion two- to fourfold. Sangivamycin at the same concentration that inhibited PKC translocation by 52% completely inhibited PTH-stimulated 1,25(OH)₂D₃ secretion. The present studies indicate that the phospholipase C/PKC pathway may mediate PTH stimulation of mammalian renal proximal tubule 1,25(OH)₂D₃ secretion. (*J. Clin. Invest.* 1992. 90:2278–2283.) Key words: rat • proximal tubules • kidney • protein kinase A • cAMP • staurosporine

Introduction

PTH maintains blood ionized Ca within a narrow normal range through its actions on Ca transport in kidney, bone, and intestine (1). PTH stimulates intestinal Ca transport indirectly

through stimulation of 1,25(OH)₂D₃ synthesis in proximal tubule cells. PTH-stimulated 1,25(OH)₂D₃ biosynthesis has been studied extensively in experimental animals in vivo (2–5) and in vitro (6–10) using kidney slices, homogenates, and isolated tubule cells. In these several models, PTH and cAMP increase 1,25(OH)₂D₃ synthesis through activation of the renal proximal tubule mitochondrial 25-hydroxyvitamin D-1 alpha-hydroxylase (1-OHase)¹ (5–7, 9–13).

PTH control of 1-OHase activity involves hormone binding to its specific plasma membrane receptor on the surface of renal proximal tubules (14, 15) and generation of intracellular second messenger signals (16). Several lines of evidence suggest that cAMP-mediated events are important as effectors of PTH stimulation of 1-OHase activity, including: PTH increases proximal tubule cAMP production (17, 18); infusion of cAMP into vitamin D-deficient rats increases conversion of tritium-labeled 25-hydroxyvitamin D₃ (25 OHD₃) to 1,25(OH)₂D₃ (19); and in vitro addition of cAMP and its dibutyryl analogue increase proximal tubule 1,25(OH)₂D₃ synthesis and 1-OHase activity (6, 11–13).

Recent evidence suggests that signal transduction systems other than cAMP may mediate one or more of the several PTH actions on renal proximal tubules. First, in proximal tubule cells in culture, kidney cell lines and proximal tubule plasma membranes (16, 20–22), PTH activates plasma membrane phospholipase C (PLC) with rapid intracellular accumulation of IP₃ and diacylglycerol (DAG). These effectors elevate cytosolic Ca (18, 23) and activate protein kinase C (PKC) (24). Second, there is a several log dose disparity between the dose of PTH that causes half-maximal inhibition of proximal tubule Na-dependent phosphate transport and the half-maximal stimulation of cAMP production (25, 26). Third, pharmacological inhibitors of PKC may block PTH inhibition of Na-dependent phosphate transport (27). The above studies indicate that PTH actions may be mediated by other signaling systems in addition to the cAMP/protein kinase A (PKA) pathway.

PLC/PKC mediation of PTH-stimulated 1,25(OH)₂D₃ production has been explored using pharmacologic stimulators and inhibitors of PKC. Phorbol esters that stimulate PKC may either inhibit (28, 29) or stimulate (30) in vitro 1,25(OH)₂D₃ production. Recently, we have shown that PKC inhibition by staurosporine blocks the PTH-mediated rise in 1,25(OH)₂D₃ secretion by perfused proximal tubules (30). In the present study, direct measurements of PKC and PKA activities, their dose-dependent activation by PTH and highly selective inhibition of PKC were used to explore the role of the cAMP/PKA

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1. Abbreviations used in this paper: CS, cytosolic; 1-OHase, 25-hydroxyvitamin D-1 alpha-hydroxylase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; rPTH 1-34, rat PTH 1-34 fragment; SM, solubilized membrane.

and PLC/PKC pathways in PTH-mediated $1,25(\text{OH})_2\text{D}_3$ production by rat renal proximal tubules.

Methods

Animals. Male Sprague Dawley rats weighing 100–125 g were fed a normal Ca diet containing 1.2% Ca, 0.9% P, 0.24% Mg, and vitamin D_3 2.2 IU/g.

Preparation of proximal tubules. Rats were placed under deep ether anesthesia and exsanguinated via the abdominal aorta. Both kidneys were removed, bisected, and washed in ice cold saline. Cortical slices were incubated in the presence of collagenase (type I; Worthington Biochemical Corp., Freehold, NJ) for 45 min at 37°C . The collagenase digest was then washed in ice cold Krebs-Henseleit (Pharmacia, Inc., Piscataway, NJ) buffer, pH 7.4, and a fraction enriched in proximal tubules was obtained by Percoll density gradient centrifugation as previously described (31).

PKC assay. Proximal tubule cytosolic and membrane fractions were prepared as described previously (32). The cytosolic (CS) fraction was saved and the pellet suspended in 5 ml of homogenization buffer A (pH 7.2) containing (mM): Tris-HCl, 20; EDTA, 0.5; EGTA, 20; benzamidine, 0.5; 2-mercaptoethanol, 5; PMSF, 2; leupeptin, 10 $\mu\text{g}/\text{ml}$; and 0.3% Triton X-100. The suspension was centrifuged at 100,000 g for 60 min and the supernatant saved as the solubilized membrane (SM) fraction. PKC-enriched CS and SM fractions were obtained by elution over DEAE-cellulose columns (Poly-prep; Bio-Rad Laboratories, Richmond, CA) with 0.08 M NaCl in buffer A.

PKC activity in the CS and SM fractions was determined using a histone phosphorylation assay as described by Kraft et al. (33) as modified by Wali et al. (32). To initiate the reaction, 50 μl of enriched fraction (10–25 μg protein) was incubated in 100 μl of reaction buffer (pH 7.2, containing [mM]: Tris-HCl, 20; MgCl_2 , 3; and 1 μCi [γ - ^{32}P]-ATP, 0.025) in the presence or absence of CaCl_2 1.83 mM and phosphatidylserine 80 $\mu\text{g}/\text{ml}$. The reaction was stopped after 5 min by blotting 50 μl of the incubation mixture onto 2.5-cm squares of phosphocellulose which were immediately immersed in 10% TCA for 5 min, washed in cold water 20 min, and soaked in 95% ethanol for 5 min and diethyl ether for 5 min. Radioactivity on the phosphocellulose was determined using a beta scintillation spectrometer. Enzyme activity was calculated as the difference in phosphorylation in the presence and absence of phosphatidylserine and Ca and is expressed as a percentage of total (CS plus SM) enzyme activity.

PKA assay. Homogenates of proximal tubules were applied to DEAE-cellulose columns and the PKA-enriched fraction eluted with 0.15 M NaCl in buffer A. PKA activity was determined using a modification of the Kemptide phosphorylation assay described by Bringhurst et al. (34). Briefly, 50 μl (~ 0.5 mg protein) was incubated with 50 μl of reaction buffer (pH 7.2) containing, in mM: Tris-HCl, 20; MgCl_2 , 3; Kemptide, 0.06; and 1 μCi [γ - ^{32}P]-ATP, 0.025 in the presence or absence of 10 μM cAMP. After 30 min the reaction was stopped by blotting the reaction mixture onto phosphocellulose paper which was immediately immersed in 10% TCA for 5 min, washed in cold water for 20 min, and then soaked in 95% ethanol for 5 min and diethyl ether for 5 min. Enzyme activity is expressed as the ratio of phosphorylated substrate in the absence of added cAMP to that phosphorylated in the presence of exogenous cAMP. Basal PKA activity ratio was 0.16 ± 0.03 in the presence of 1 mM isobutylmethylxanthine, and 0.12 ± 0.04 in the absence of isobutylmethylxanthine (P , NS). Therefore, experiments were performed in the absence of isobutylmethylxanthine. Preliminary studies showed that PTH-stimulated PKA activity was detectable in dilutions of proximal tubule cell homogenates and in DEAE-cellulose purified fractions of homogenates. Therefore, DEAE-cellulose eluates were used as the source of PKA in subsequent studies.

cAMP assay. cAMP in proximal tubules was determined using a commercial radioimmunoassay kit (Biomedical Technologies, Inc., Stoughton, MA) following the directions provided.

$1,25(\text{OH})_2\text{D}_3$ assay. $1,25(\text{OH})_2\text{D}_3$ was assayed in acetonitrile extracts of perfusion effluent using a radioreceptor assay as previously

described (30, 31). Intra- and interassay coefficients of variation were 7% and 12%, respectively.

Perfusion of proximal tubules. Perfusion was performed using an Acusyst-S system (Endotronics, Inc., Minneapolis, MN) which consisted of six parallel flow-through Lucite chambers, a Haake circulating water pump to maintain buffer and tubules at 37°C , a gas diffusion block to oxygenate the buffer, a peristaltic pump to move the buffer from the reservoirs through the Lucite chambers and a fraction collector to control collection of effluent. Proximal tubules (100–200 μg tubule protein) were placed into the Lucite chambers, and buffer was pumped at 0.1 ml/min with KHS containing 2.5% BSA, 5 mM L-glutamine, and 10 μg 25-hydroxyvitamin D_3 . Rat PTH (rPTH 1-34) (10^{-11} M) with or without sangivamycin was added to some reservoirs. Effluent was collected in 5-min aliquots and $1,25(\text{OH})_2\text{D}_3$ was assayed.

Materials. [^3H] $1,25(\text{OH})_2\text{D}_3$, sp act 90–110 Ci/mmol, was purchased from Amersham Searle (Arlington Heights, IL). 25-hydroxyvitamin D_3 was a generous gift from Organon, Inc. (West Orange, NJ). $1,25(\text{OH})_2\text{D}_3$ was a generous gift from Dr. Milan Uskokovic, Roche Laboratories (Nutley, NJ). rPTH 1-34, staurosporine, and phorbol ester were purchased from Sigma Chemical Co. (St. Louis, MO). Percoll was purchased from Pharmacia Inc., and collagenase type I was purchased from Worthington Biochemical Corp. Sangivamycin was a gift from the National Cancer Institute.

Statistical analysis. Differences between group means were analyzed by t test or by analysis of variance when the means of more than two groups were being compared. Data are shown as mean \pm standard error (SEM). Differences between or among group means were considered statistically significant when P values were < 0.05 .

Results

PKC activity is readily detectable in CS and SM fractions of freshly prepared renal proximal tubules, with the majority of activity residing in CS. Addition of rPTH 1-34 (10^{-7} M) promptly translocates PKC activity from CS to SM fractions (Fig. 1A) in a time-dependent manner, with no change in total (CS plus SM) PKC activity. rPTH 1-34 causes a dose-dependent translocation of PKC activity from the CS to the solubilized plasma membrane-rich SM fraction (Fig. 1B) without an absolute change in overall PKC. Significant translocation of PKC activity occurs in the presence of physiologic concentrations of rPTH 1-34 (10^{-11} – 10^{-10} M), and SM activity exceeds CS at $\sim 10^{-10}$ M PTH.

Low levels of PKA activity are readily detectable in proximal tubule cell homogenates in the absence of exogenous cAMP. The PKA activity ratio increases linearly for 5 min and then approaches a plateau, therefore subsequent measurements were made at 3 min, during the linear portion of the increase in activity (data not shown). rPTH 1-34 increases the PKA activity ratio at 10^{-7} M, but not at lower concentrations (Fig. 2). Proximal tubule cell homogenates contain low concentrations of cAMP, and cAMP concentration increases in the presence of rPTH 1-34 10^{-7} M, but not at lower concentrations (Fig. 3).

To determine the specificity of inhibitors of PKC and PKA, proximal tubules were incubated in the presence of sangivamycin or staurosporine 30 min before the addition of rPTH 1-34 (10^{-11} M). Sangivamycin (10 μM) reduced PTH-stimulated PKC translocation by 52%, and greater concentrations (50 μM) virtually abolished PTH-stimulated PKC translocation (Fig. 4). Staurosporine failed to reduce PTH-stimulated PKC translocation at 1 nM, but at 10 nM it reduced PTH-stimulated PKC translocation by 60% (Fig. 4). Physiologic concentrations of rPTH 1-34 (10^{-11} M) failed to increase proximal tubule

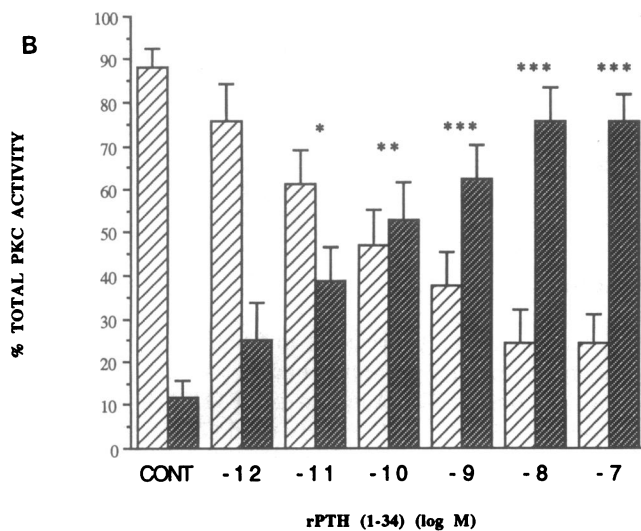
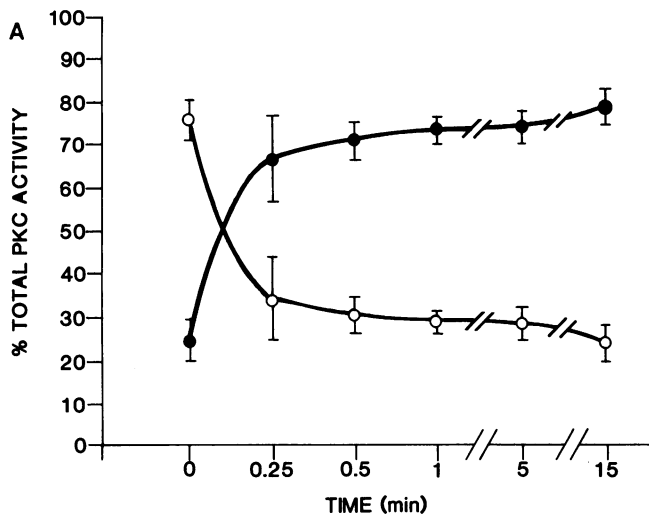


Figure 1. (A) Time course of translocation of rat proximal tubule PKC from cytosol (open circles) to solubilized membrane (closed circles) in response to rPTH 1-34 10^{-7} M. Enzyme activity is expressed as percentage of total PKC activity in each fraction. Values are mean \pm SEM for 12 determinations in three separate experiments. For cytosolic and solubilized membrane fractions, all activity after 0.5 min is significantly different from control, $P < 0.01$. (B) Dose-response of rat proximal tubule solubilized membrane (shaded bars) and cytosolic (hatched bars) PKC activity in the absence (control) and presence of rPTH 1-34 10^{-12} – 10^{-7} M for 1 min. Data are expressed as percentage of total PKC activity. Values are mean \pm SEM for 18 determinations in six separate experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs control.

PKA activity ratio (Fig. 2). Sangivamycin (10 and 50 μ M) did not alter basal PKA activity ratio at either of the concentrations that reduced PKC translocation by 52 and 95% (Fig. 5). In contrast, staurosporine inhibited basal PKA activity ratio by 62% at concentrations (10 nM) that also reduced PKC translocation (Fig. 5).

Low levels of $1,25(\text{OH})_2\text{D}_3$ are detectable in the effluent of perfused proximal tubules in the absence of 25-hydroxyvitamin D_3 substrate (data not shown). Addition of substrate to the perfusion medium increases $1,25(\text{OH})_2\text{D}_3$ in the effluent within 5–10 min (data not shown), and approaches a plateau by 20–30 min (Fig. 6). Addition of 10^{-11} M rPTH to the peri-

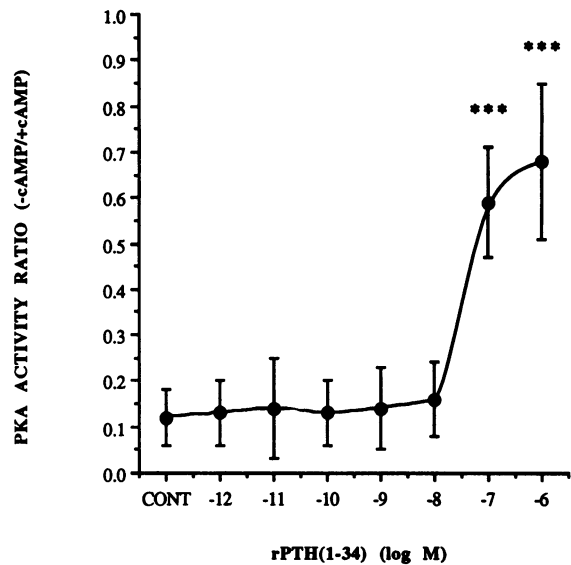


Figure 2. Dose response of rat proximal tubule homogenate cAMP-dependent PKA activity ratio in the absence (control) and presence of rPTH 1-34 10^{-12} – 10^{-6} M for 3 min. Values are mean \pm SEM for 18 determinations in six separate experiments. *** $P < 0.001$ vs control.

fusate increased $1,25(\text{OH})_2\text{D}_3$ secretion more rapidly and, by 15 min, exceeded $1,25(\text{OH})_2\text{D}_3$ levels secreted in the absence of rPTH. Addition of sangivamycin (10 or 50 μ M) to the perfusate did not significantly depress basal $1,25(\text{OH})_2\text{D}_3$ secretion, but did completely abolish the PTH-induced rise in $1,25(\text{OH})_2\text{D}_3$ secretion (Fig. 6).

Discussion

The primary observations of this study support a role for the PLC/PKC pathway in mediating PTH stimulation of $1,25(\text{OH})_2\text{D}_3$ secretion by mammalian renal proximal tubule

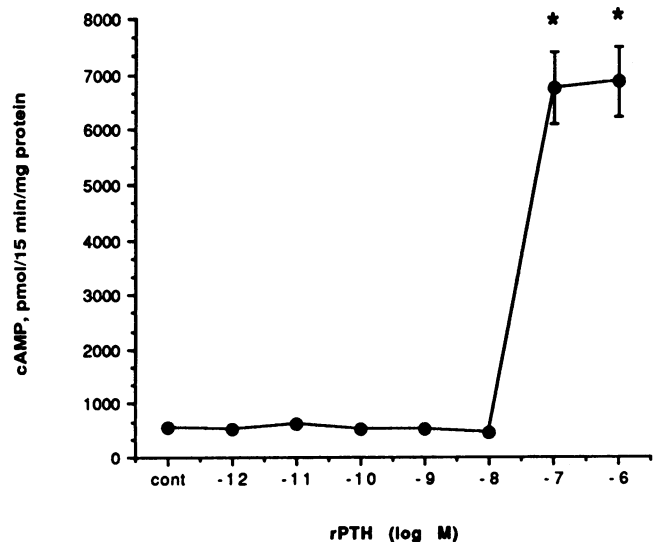


Figure 3. Dose response of rat proximal tubule cAMP production in the absence (control) and presence of rPTH 1-34 10^{-12} – 10^{-6} M for 3 min. Values are mean \pm SEM for 12 determinations in four separate experiments. * $P < 0.001$ vs control.

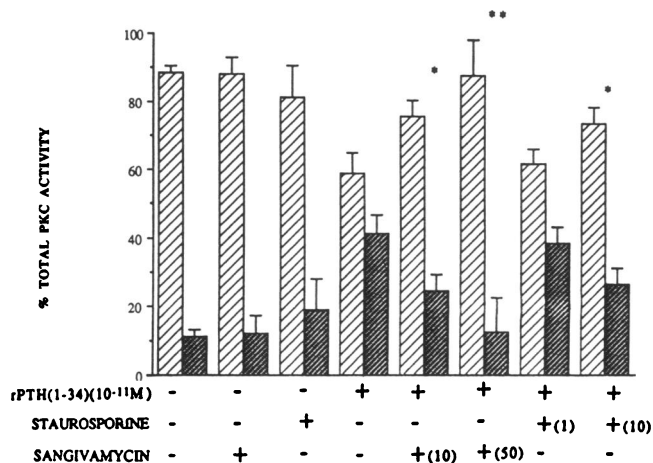


Figure 4. Rat proximal tubule PKC activity in the cytosolic fraction (hatched bars) and solubilized membrane fraction (shaded bars) in the absence (-) and presence (+) of rPTH 1-34 10^{-11} M for 1 min. In some samples, either sangivamycin (10 or 50 μ M) or staurosporine (1 or 10 nM) were added 30 min before PTH. Enzyme activity is expressed as in Fig. 1. Values are mean \pm SEM for 12 determinations in three separate experiments. * $P < 0.05$; ** $P < 0.01$ vs rPTH 1-34 alone.

cells. We conclude that PTH-mediated increases in $1,25(\text{OH})_2\text{D}_3$ secretion require an intact PLC/PKC pathway. Three lines of evidence were developed that support our conclusion. First, while previous studies show that direct stimulation in vitro by cAMP increases 1-OHase activity (6, 10–13), the present study demonstrates a discrepancy between the concentration-response curves for PTH-stimulated cAMP generation and PKA activity and the hormone's effects on proximal tubule $1,25(\text{OH})_2\text{D}_3$ secretion. Concentrations of rPTH 1-34 that are too low to detect increases in cAMP content or activation of PKA increase tubule $1,25(\text{OH})_2\text{D}_3$ secretion. Second, these concentrations of PTH ($< 10^{-10}$ M) that are well below the concentrations required to elevate measurably cAMP production or activate PKA are sufficient to activate PKC and

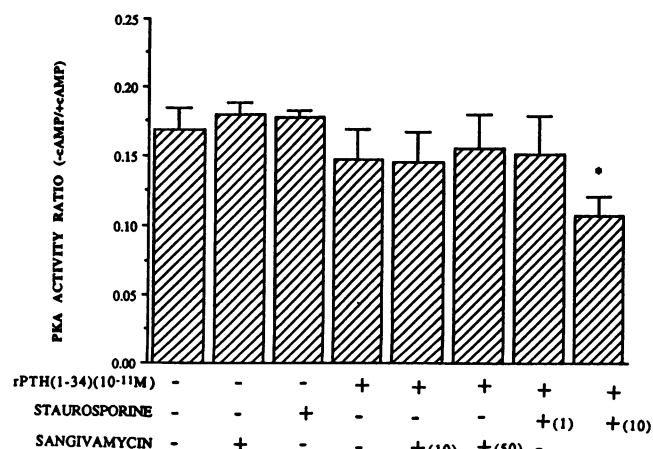


Figure 5. Rat proximal tubule homogenate PKA activity ratio in the absence (-) and presence (+) of rPTH 1-34 10^{-11} M alone or after 30-min pretreatment with sangivamycin or staurosporine (concentrations are as in Fig. 4). Enzyme activity is expressed as in Fig. 2. Values are mean \pm SEM for 12 determinations in three separate experiments. * $P < 0.05$ vs rPTH 1-34 alone.

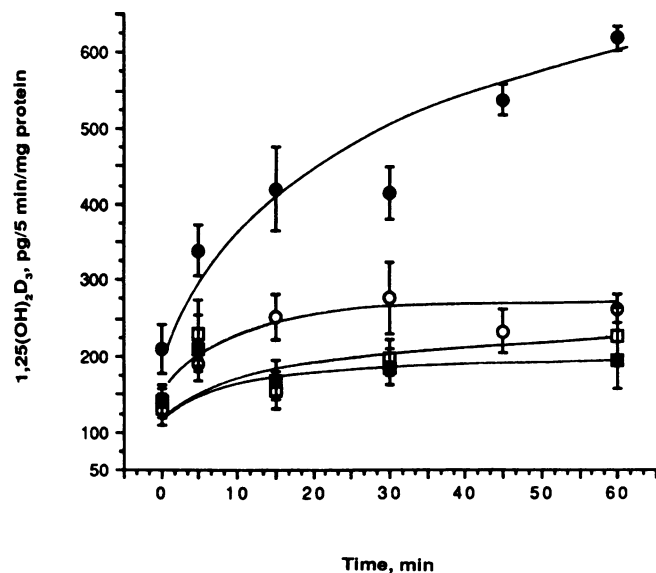


Figure 6. Proximal tubules from rats fed a normal Ca diet were perfused (see Methods) with either 25OHD_3 alone (solid squares); or 25OHD_3 plus rPTH 1-34 (10^{-11} M, closed circles); or 25OHD_3 plus rPTH (10^{-11} M) after 30 min of incubation with sangivamycin (10 μ M, open circles); and 50 μ M, open squares). Values are mean \pm SEM for three to seven observations per time point for each group. For rPTH 1-34 alone vs other groups: $P < 0.01$ for 5–30 min; and $P < 0.001$ for 45–60 min.

increase $1,25(\text{OH})_2\text{D}_3$ secretion. The lower concentrations of rPTH 1-34 that activate PKC also stimulate PLC with concomitant increases in other components of the PLC/PKC pathway: IP_3 , diacylglycerol, and cytosolic Ca (16, 20, 21, 35).

A third line of evidence for a role for PKC in PTH actions on $1,25(\text{OH})_2\text{D}_3$ production comes from the studies using a selective inhibitor of PKC. Although PKC inhibition by staurosporine has been widely used as a probe to explore PKC participation in biologic processes (20, 27, 30, 33, 36), the present study clearly shows that concentrations of staurosporine that inhibit PKC also inhibit basal PKA activity. The inability of staurosporine to act as a selective inhibitor of PKC is in contrast to results observed with the nucleoside inhibitor sangivamycin, which induced a 52 and 95% inhibition of PKC activity at concentrations that did not inhibit PKA. While the present study did not test whether greater concentrations of sangivamycin would inhibit proximal tubule PKA, a previous study (37) reported 50% inhibition of central nervous system-derived PKA at concentrations that were five times greater than that required for 50% inhibition of PKC. The mechanism of sangivamycin action is complex, as it acts as a competitive inhibitor of PKC activity with respect to ATP and a noncompetitive inhibitor with respect to histone and the lipid co-factors diacylglycerol and phosphatidylserine (37).

The present study clearly demonstrates that proximal tubule cells contain high levels of cytosolic or non-membrane-associated PKC activity. Our observations are similar to a previous measure of PKC activity in cortical tubule cells (27), which demonstrated rapid activation by PTH, detectable within seconds, followed by a decline in activity. Like other activators of PKC (20, 33, 38), PTH translocated PKC from the cytosol to the SM fraction. Over the range of concentrations used in the present study (10^{-12} – 10^{-7} M), rPTH 1-34 did

not alter total PKC activity (CS plus SM), but progressively increased PKC transfer from CS to SM fraction.

The PKC activity we measured may represent the aggregate of one or more PKC isoforms. Because maximal activity of each isoform may vary depending upon assay conditions, and because the scheme we used to purify PKC may not have eluted all PKC isoforms, it is not possible to infer from our measurements which isoform(s) is (are) involved in the renal proximal tubule response to PTH.

Because cAMP accumulation may not accurately reflect the effect of intracellular cAMP, and because PKA activity may increase with minimal or undetectable changes in cAMP (36), PKA activity was measured directly. The dose-response curve for PTH activation of PKA was similar to that for cAMP accumulation, and fails to explain the PTH concentration difference between cAMP generation and activation of 1,25(OH)₂D₃ secretion. The present study and previous reports of cAMP stimulation of 1-OHase activity are consistent with the presence of a cAMP-dependent pathway activated by pharmacologic concentrations of cAMP and PTH and located either in a subset or in all proximal tubule cells that contain the 1-OHase enzyme complex.

The present observations are in agreement with previous studies of in vitro addition of PTH to chick renal tubules (6, 11), guinea pig cortical tubules (9), and rat renal cortical slices (12) in which PTH increases 1,25(OH)₂D₃ synthesis within minutes. Previous reports using chick kidney tubule cells in primary culture described phorbol ester inhibition of PTH induced 1,25(OH)₂D₃ production (28). The apparent discrepancy between the results of Henry and colleagues using cell culture (28, 29) and the present study and another recent report from our laboratory (30) may be due to our use of the perfusion system which removes 1,25(OH)₂D₃ from the tubule cells and thus prevents product inhibition of the 1-OHase. Also, there are differences between the studies of Henry et al. (28, 29) in length of exposure to PTH and phorbol ester. Henry exposed cultured chick kidney cells to phorbol ester for several hours, which may have desensitized the PTH receptor to PTH (39) and abolished PKC activity through a direct interaction with the enzyme's phorbol ester binding site (40) or through depletion of PKC.

In summary, PTH activation of either of two separate, parallel second messenger pathways results in the same biologic action to increase 1,25(OH)₂D₃ secretion by proximal tubule cells. That PTH can activate both pathways has raised the question of which pathway may be favored under physiologic conditions. The present findings that physiologic concentrations of PTH activate PKC and that selective inhibition of PKC blocks PTH stimulation of 1,25(OH)₂D₃ secretion, strongly suggest that PTH action may be mediated preferentially via the PLC/PKC pathway. While cAMP and PKA do not appear to be responsive to physiologic concentrations of PTH, the present study does not exclude a role for the cAMP/PKA pathway in physiologic regulation of PTH-stimulated 1,25(OH)₂D₃ production. It remains to be unequivocally determined whether the results of these studies using in vitro systems are operative in the proximal tubule in vivo.

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