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Regulation of nucleobase transport in LLC-PK$_1$ renal epithelia by protein kinase C

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Abstract

The involvement of protein kinase C (PKC) in the regulation of Na^+-dependent and -independent hypoxanthine transport was investigated by exposing confluent monolayers of LLC-PK$_1$ renal epithelia cells to the PKC activator, phorbol 12-myristate 13-acetate (PMA). Chronic exposure (> 2 h) of LLC-PK$_1$ monolayers to 16 nM PMA resulted in ≈ 75% inhibition of Na^+-dependent hypoxanthine influx occurring maximally at 8 h and persisting for 72 h. In contrast, PMA had little effect on Na^+-independent hypoxanthine influx at 8 h, but longer exposure resulted in stimulation of influx (≈ 3-fold) that peaked at 24 h and thereafter declined to control levels at 72 h. The effects of PMA were dose-dependent and were associated with changes in V_{max} of transport (2–4-fold) with no significant change in apparent K_m. 4α-Phorbol, a phorbol ester that does not activate PKC, had no effect on hypoxanthine transport by LLC-PK$_1$ cells. The diacylglycerol kinase inhibitor, R59022 (10 μM), partially inhibited (28%) Na^+-dependent hypoxanthine influx. In addition, the PMA-induced effects on hypoxanthine transport were reversed by Ro-31-8220 (1 and 5 μM) and calphostin C (50 nM), potent and selective inhibitors of PKC. The increase in Na^+-independent hypoxanthine influx following exposure to PMA was blocked by the protein synthesis inhibitor, cycloheximide (20 μM), and correlated with an increase in LLC-PK$_1$ cell proliferation. The PMA-induced decrease in Na^+-dependent hypoxanthine transport was independent of PMA effects on cell proliferation and not dependent on protein synthesis. These results are consistent with the proposal that the PMA-induced effects on hypoxanthine transport are due to PKC activation.

Keywords: Hypoxanthine transport; Sodium ion/nucleobase cotransport; Nucleobase cotransport; Facilitated-diffusion nucleobase transport; Phorbol ester; Phorbol 12-myristate 13-acetate; Diacylglycerol

1. Introduction

Renal fractional clearance measurements of hypoxanthine and xanthine in human adults [1–3] indicate that these compounds undergo net reabsorption in the mammalian kidney. In addition, similar measurements in mice have shown that the pyrimidine nucleobase analogue, 5-fluorouracil, is also actively reabsorbed in the kidney [4]. More recent studies with the proximal tubular epithelial cell line (LLC-PK$_1$) [5], and brush-border membrane vesicles isolated from the cortex of guinea pig kidney [6], have demonstrated that the purine nucleobases, hypoxanthine and guanine, and the pyrimidine bases, uracil, 5-fluorouracil and thymine, are actively transported across the apical membrane by a high-affinity Na^+ cotransport mechanism. LLC-PK$_1$ cells also possess a low-affinity facilitated-diffusion nucleobase carrier in the basolateral membranes [5]. There is currently no information available regarding the molecular basis of these transport processes, nor their regulation.

Protein kinase C (PKC) plays a central role in receptor-mediated signal transduction, most notably in the control of cell proliferation and differentiation [7]. There are at least ten isoforms of the enzyme that exhibit differential tissue expression and can be divided into three groups on the basis of their requirements for activation [8]. The first group of conventional or classical PKC (cPKC) isoforms (α, βI, βII and γ) are dependent on phosphatidylserine and Ca^{2+} for activation, and a number of other phospholipid metabolites such as DAG and arachidonic acid, the latter being endogenous activators generated by the hydrolysis of membrane phosphoinositides by phospholipase C

Abbreviations: PMA, phorbol 12-myristate 13-acetate; OAG, 1-oleoyl-2-*sn*-glycerol; DAG, diacylglycerol; HIFCS, heat inactivated fetal calf serum; NMG^+, *N*-methyl-D-glucamine; PKC, protein kinase C.

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[7,8]. The second group consists of four new PKC (nPKC) isoforms (δ, ε, η and θ) that are Ca^{2+}-independent, but still require phosphatidylserine and DAG for activity. The most recently identified group contains two atypical PKC (aPKC) isoforms (ζ and λ) that have not been completely characterized, but appear to be both Ca^{2+} and DAG-independent [8]. The tumour-promoting phorbol esters, such as PMA, are also potent activators of the cPKC and nPKC isoforms that bind specifically to these molecules [7–10] at a site identical to or overlapping with the DAG binding site [11]. PKC has been shown to be present in the epithelial cells of the mammalian renal proximal tubule [12–14], and has been implicated in the physiological regulation of both brush-border (e.g. Na^+/P_i cotransport [15]) and basolateral (e.g. Na^+,K^+-ATPase [16]) membrane transport processes in these cells.

The objective of this study was to evaluate the role of protein kinase C in the regulation of renal nucleobase transport by analyzing the effect of PMA on Na^+-dependent and -independent hypoxanthine transport by LLC-PK_1 cells. The results presented herein provide evidence to implicate protein kinase C in the long-term physiological regulation of the expression of nucleobase transporters in the mammalian kidney.

A preliminary report of some of these results has been published [17].

2. Materials and methods

2.1. Cell culture

LLC-PK_1 cells were originally obtained from the American Type Culture Collection at serial passage 199, and experiments were performed using cells between passages 212 and 235. Cells were grown in Dulbecco's modified Eagle's medium and subcultured for serial passage and experimental use (on 24-well tissue culture plates) as previously described [5], except that cells were seeded at a cell density that was twice that achieved at confluency ($2.5 \cdot 10^5$ cells/cm^2). A superconfluent cell density was employed in the present study to promote differentiation and minimize the stimulus for cell division [18]. Cells used in the present study were free of mycoplasma as detected using Hoechst 33258 stain.

2.2. Hypoxanthine transport measurements

The influx of [^{3}H]hypoxanthine by LLC-PK_1 cells was determined at room temperature (22°C) as previously described [5], except the media also contained 0.8 mM phosphate and 5.5 mM glucose. The ratio ($27 \pm 1{:}1$, $n = 6$) of cellular protein content, determined by the method of Lowry et al. [19], to total DNA, measured by the fluorometric method of Fiszer-Szafarz et al. [20], was not significantly altered by the various treatments (PMA, staurosporine, cycloheximide, etc.) described in the present paper, and thus, the results of uptake experiments are expressed per mg of protein.

2.3. Flame photometry

The total intracellular Na^+ and K^+ content of LLC-PK_1 monolayers was determined by flame photometry. Cells grown in 6-well plates were incubated with 3 ml of DMEM/HIFCS containing either 16 or 1600 nM PMA or 0.08% (v/v) DMSO (PMA solvent as control) for 24 h at 37°C. The culture medium was then aspirated and cell monolayers rinsed five times with 3 ml of an ice-cold medium containing (in mM): 150 tetramethylammonium chloride (TMA-Cl), 1 $MgSO_4$, 1 $MgCl_2$, 1 EGTA, and 10 Hepes (titrated to pH 7.4 with TMA-OH). Cells were lysed by the addition of 1 ml of ice-cold 10% (w/v) PCA and left on ice for 15 min. Acid insoluble material was removed by brief centrifugation in a microcentrifuge (13 000 $\times g$ for 2 min), and the clear supernatant was read directly in a Corning 410 flame photometer against standards prepared in 10% (w/v) PCA. The results were normalized to the concentration of acid-insoluble protein of each sample.

2.4. [^{3}H]Thymidine incorporation

The incorporation of [^{3}H]thymidine into the DNA of untreated and PMA-treated LLC-PK_1 cells was used to compare rates of cell proliferation. On the day of experiments, cells were incubated in serum containing DMEM medium at 37°C either in the presence of 16 and 1600 nM PMA or it's solvent DMSO (0.08% (v/v)). A duplicate set of cells from the same batch was established for total DNA determination. The cells were refed 8 or 24 h later with fresh culture medium containing 2 μCi/ml [^{3}H]thymidine. Incorporation (for 2 h at 37°C) was terminated by aspirating the medium and washing monolayers three times with 1 ml aliquots of ice-cold transport medium. Cell monolayers were then covered with 0.2 ml of ice-cold 4% (w/v) perchloric acid. Wells were scraped to suspend precipitated monolayers, which were then transferred to 1.5 ml microcentrifuge tubes. The precipitate was pelleted (13 000 $\times g$ for 2 min), washed once with 4% (w/v) perchloric acid and dissolved in 0.2 ml of 0.5 M NaOH. Radioactivity in the samples was measured by liquid scintillation counting. The results of incorporation experiments were expressed as dpm [^{3}H]thymidine per μg of total DNA. Incorporation of [^{3}H]leucine (2 μCi/ml) into the acid-insoluble protein of LLC-PK_1 cells incubated in the presence and absence of 16 nM PMA and 20 μM cycloheximide for 24 h at 37°C was determined as described above for incorporation of [^{3}H]thymidine.

2.5. Data analysis

All transport experiments were carried out in triplicate. The errors given in the tables and figures are standard deviations where data from a representative experiment are presented or S.E.M. when the mean results from three or more separate experiments are shown. In least-square fits to the data, points were weighted according to the inverse of their relative experimental errors. Non-linear regression analysis of data were performed using the computer program Enzfitter (Elsevier Biosoft). Statistical significant differences between data were determined using Student's *t*-test. Statistical significance was defined as a P value of less than 0.05.

2.6. Materials

Cell culture reagents and plasticware were purchased from Gibco, Paisly, UK and Flow Laboratories, Glasgow, UK. [G-^{3}H]Hypoxanthine (3.8 Ci/mmol), [U-^{14}C]sucrose (0.56 Ci/mmol), [6-^{3}H]thymidine (25 Ci/mmol) and [4,5-^{3}H]leucine (78 Ci/mmol) were obtained from Amersham International (Amersham, UK). PMA, cycloheximide and actinomycin D were obtained from Sigma (Poole, Dorset, UK). 4α-Phorbol, staurosporine, calphostin C, Ro-31-8220 and DAG kinase inhibitor R59022 were purchased from Calbiochem-Novabiochem, Nottingham, UK. All other reagents were analytical grade.

3. Results

3.1. Time- and dose-dependence of PMA effects on hypoxanthine transport by LLC-PK$_1$ cells

Preliminary experiments demonstrated that both Na^+-dependent and -independent hypoxanthine transport rates were stable in cell monolayers cultured for between 3 and 24 days (data not shown). Thus, in order that differentiated cell characteristics be examined in the present study, highly confluent cell monolayers were used, i.e. between 10 and 20 days in culture. We have previously shown that initial rates of hypoxanthine transport in LLC-PK$_1$ cells can be estimated using a 60-s time interval [5]. Control experiments established that in the present study hypoxanthine influx by PMA-treated cells was also linear for up to 2 min and thus initial rates of hypoxanthine transport were measured using a 60-s time interval. The effect of 16 and 1600 nM PMA on the initial rate of Na^+-dependent and -independent hypoxanthine (2 μM) influx by LLC-PK$_1$ cells as a function of the time of exposure to PMA is shown in Fig. 1. Incubation of cells with 16 or 1600 nM PMA had no detectable effect on hypoxanthine influx for time intervals of exposure of < 2 h (i.e. 5, 10, 20, 30 and 60 min; data not shown). However, after prolonged incubation with PMA (Fig. 1), Na^+-dependent hypoxanthine transport decreased (as compared to the control incubation with 0.08% (w/v) DMSO (PMA solvent)). Inhibition was first apparent at 4 h, maximal at 8 ($\approx$ 40%) and 16 h ($\approx$ 42%) for 16 and 1600 nM PMA, respectively, and persisted with a similar magnitude for at least 48 h. In contrast, 16 nM PMA had little effect on Na^+-independent hypoxanthine influx at 8 h, but at longer time intervals resulted in a stimulation of transport that was maximal at about 24 h ($\approx$ 3-fold), and thereafter decreased to control levels by 72 h. Interestingly, 1600 nM PMA had no effect on Na^+-independent hypoxanthine influx (Fig. 1, lower panel). Simple diffusion of hypoxanthine into LLC-PK$_1$ cells (estimated in the presence of NMG^+ and an excess of nonlabelled

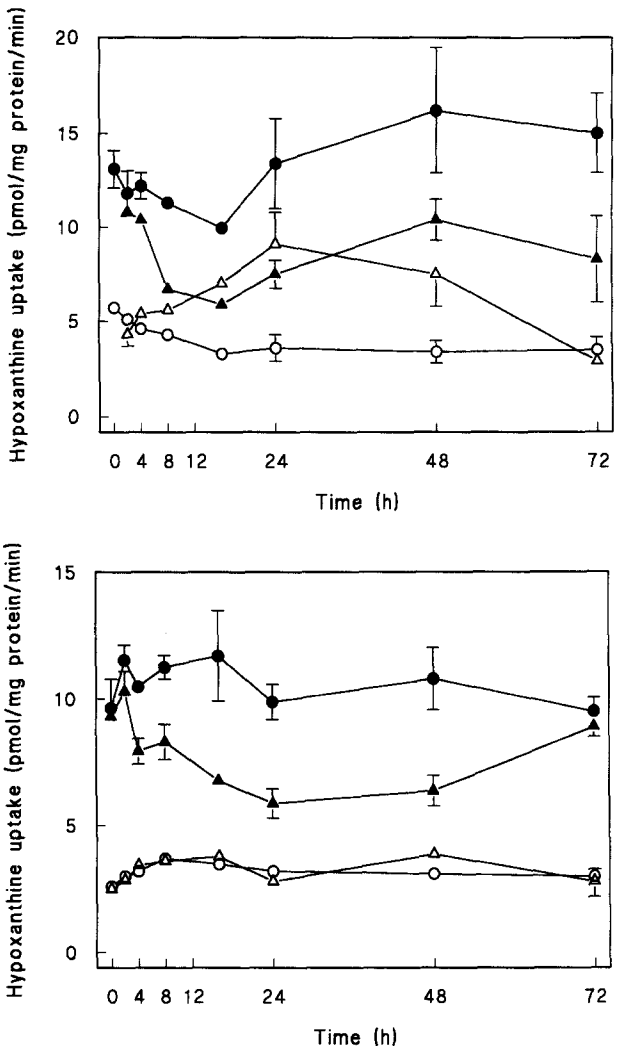

Fig. 1. Time-course of the effect of PMA on Na^+-dependent and Na^+-independent hypoxanthine influx by LLC-PK$_1$ cells. Cells were incubated in serum containing medium either in the presence of PMA (16 nM, upper panel or 1600 nM, lower panel; triangles) or 0.08% (v/v) DMSO (circles) for the indicated time intervals. The initial rate of [^{3}H]hypoxanthine influx (1 μM, 1 min) was then determined at 22°C in the presence of 140 mM NaCl (total uptake) or NMG^+ (○, △). Na^+-dependent hypoxanthine uptake (●, ▲) was obtained by subtracting the rate in the presence of NMG^+ from the total uptake values.

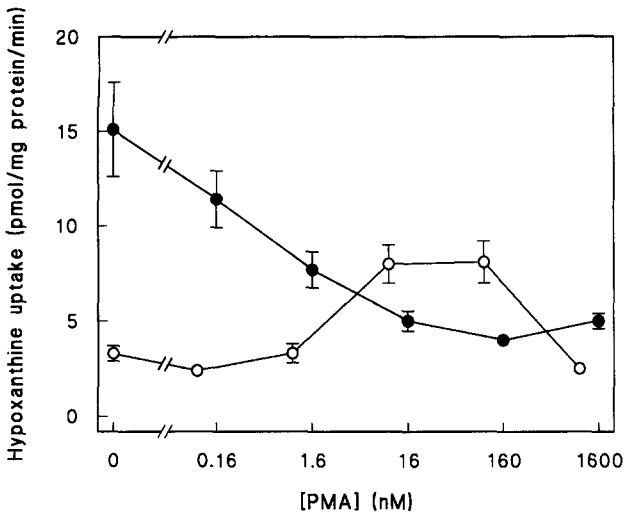

Fig. 2. Concentration dependence of the action of PMA on hypoxanthine influx by LLC-PK$_1$ cells. Cells were incubated for 24 h at 37°C with PMA at the indicated concentrations. The initial rate of 2 μM hypoxanthine influx via the Na^+-dependent pathway (●) and the Na^+-independent route (○) were determined as described in the legend to Fig. 1. Values represent means ± S.E.M. of three separate experiments.

hypoxanthine (2 mM)) was not affected by PMA treatment (data not shown). The % contribution of the simple diffusion component of hypoxanthine influx in the presence and absence of Na^+ was on average 1 and 5%, respectively.

Both the inhibition of Na^+-dependent and the stimulation of Na^+-independent hypoxanthine transport after 24 h exposure to PMA were dose-dependent (Fig. 2). Inhibition of Na^+-dependent hypoxanthine transport was observed at 0.16 nM PMA (≈ 33% of maximum inhibition), the lowest concentration tested, and was maximal at 160 nM. In contrast, no stimulation of Na^+-independent hypoxanthine transport was observed at 0.16 and 1.6 nM PMA. Maximal stimulation was obtained at 16 nM PMA. In agreement with the data given in Fig. 1 (lower panel), the dose–response curve for stimulation of Na^+-independent hypoxanthine transport by PMA was biphasic showing no stimulation of uptake at 1.6 μM.

To test whether the PMA-induced inhibition of Na^+-dependent hypoxanthine transport was the consequence of a decrease in the Na^+ gradient across the membrane, the intracellular content of Na^+ in LLC-PK$_1$ cells was measured following PMA treatment. Table 1 shows that the intracellular Na^+ content of PMA-treated cells was not significantly different to that of control cells. These results were further supported by experiments that examined the effect of amiloride. This compound is an inhibitor of Na^+/H^+ exchange, a process that has been reported to be activated by phorbol esters [21]. Amiloride (100 μM) by itself or in the presence of PMA (16 nM) had no significant effect on hypoxanthine transport (values for PMA-treated cells in the presence and absence of amiloride were 2.4 ± 0.1 and 2.1 ± 0.3 pmol/mg protein per min, respectively, for Na^+-dependent, and 7.9 ± 0.2 and 8.3 ± 0.2 pmol/mg protein per min, respectively, for Na^+-independent hypoxanthine influx).

Table 1
Intracellular ion content of LLC-PK$_1$ cells after treatment with PMA

	[Intracellular ion] (nmol/mg protein)	
	Na^+	K^+
Control	100 ± 30	660 ± 30
PMA-treated (nM)		
16	83 ± 13	690 ± 50
1600	72 ± 12	680 ± 40

Cells were incubated for 24 h at 37°C either in the presence of 16 or 1600 nM PMA, or 0.08% (v/v) DMSO (PMA solvent as control). The intracellular ion content of LLC-PK$_1$ cells was then determined as described in Section 2. Data are the means ± S.E.M. of three or four separate experiments.

The specificity of the PMA-induced changes in hypoxanthine transport by LLC-PK$_1$ monolayers was tested by using the non-protein-kinase-C-stimulating phorbol ester, 4α-phorbol. Fig. 3 shows that 4α-phorbol (27 and 2700 nM) failed to stimulate Na^+-independent hypoxanthine transport. Similarly 2.7 μM 4α-phorbol had no significant effect on Na^+-dependent hypoxanthine influx. At 100-fold lower concentrations of 4α-phorbol, this compound inhibited slightly (20%) Na^+-dependent hypoxanthine influx, but this inhibition was not statistically significant at the level of $P > 0.05$.

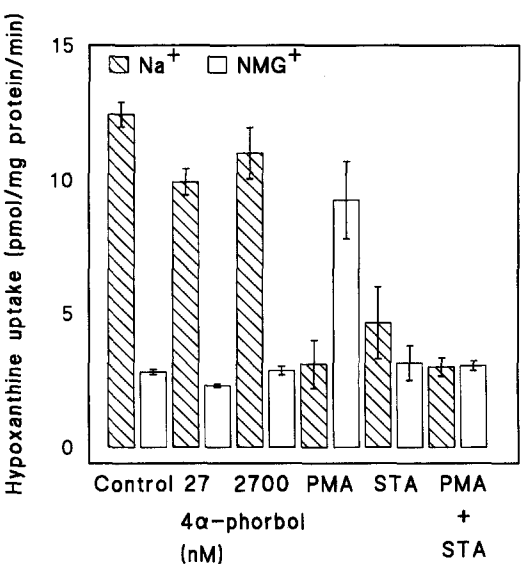

Fig. 3. Effect of 4α-phorbol and staurosporine on hypoxanthine transport by LLC-PK$_1$ cells. Cells were incubated for 24 h at 37°C with 0.08% (v/v) DMSO (control), or 4α-phorbol (27 or 2700 nM), or 16 nM PMA, or 100 nM staurosporine (STA), or 16 nM PMA plus 100 nM staurosporine (PMA + STA). Hypoxanthine influx (2 μM) at 22°C was measured in the presence of 140 mM NaCl (total uptake) or NMG^+ (open bars). Na^+-dependent hypoxanthine uptake (hatched bars) was obtained by subtracting the rate of influx in the presence of NMG^+ from the total uptake values. Means ± S.E.M. of three separate experiments are shown.

Table 2
Effect of the DAG kinase inhibitor, R59022, and the protein kinase C inhibitors, calphostin C and Ro-31-8220, on hypoxanthine transport by LLC-PK$_1$ cells

	Treatment	Hypoxanthine influx (% of Control)			
		Na^+-dependent	P	Na^+-independent	P
Expt. 1	R59022 (10 μM)	72 ± 6	S($P < 0.02$)	105 ± 11	NS
Expt. 2	PMA (16 nM)	7 ± 0.1	S	460 ± 20	S
	Calphostin C (50 nM)	79 ± 17	NS	79 ± 7	NS
	PMA (16 nM) + Calphostin C (50 nM)	49 ± 6	S [a]	330 ± 31	S [a]
Expt. 3	PMA (16 nM)	22 ± 5	S	270 ± 5	S
	Ro-31-8220 (1 μM)	94 ± 1	NS	88 ± 13	NS
	Ro-31-8220 (5 μM)	91 ± 9	NS	94 ± 18	NS
	PMA (16 nM) + Ro-31-8220 (1 μM)	34 ± 5	S [a]	180 ± 19	S [a]
	PMA (16 nM) + Ro-31-8220 (5 μM)	66 ± 7	S [a]	120 ± 31 [a]	S [a]

Confluent monolayers of LLC-PK$_1$ were incubated with the compounds indicated for 24 h and then initial rates of Na^+-dependent and -independent hypoxanthine influx (2 μM) were determined at 22°C. In the case of the calphostin C experiment, the cells were incubated in the presence of light. The data are expressed as a percentage of the control hypoxanthine flux rate (7.9 ± 0.19, 8.5 ± 0.65 and 7.6 ± 0.35pmol/mg protein per min for the Na^+-dependent flux and 1.3 ± 0.13, 2.9 ± 0.4 and 1.6 ± 0.14 pmol/mg protein per min for the Na^+-independent flux, for experiments 1, 2 and 3, respectively). Statistical significance was defined as a P value of less than 0.05 unless indicated and in most cases the test condition was compared with the control.

[a] The data set was compared with the PMA-treated cells and not with the control.

3.2. Effect of the DAG kinase inhibitor, R59022

Table 2 shows that incubation with R59022 (10 μM), which blocks the inactivation of endogenous DAG, inhibited Na^+-dependent hypoxanthine uptake in LLC-PK$_1$ cells by ≈ 28%. R59022 had no detectable effect on Na^+-independent hypoxanthine influx. Incubation of LLC-PK$_1$ cells with OAG, a permeant DAG analogue [22], at 50 or 125 μM for 24 h had no effect on hypoxanthine influx via either the Na^+-dependent or -independent routes (data not shown).

3.3. Effects of PMA on the kinetic constants for hypoxanthine transport

Results presented in Table 3 demonstrated that PMA-induced effects on both Na^+-dependent and -independent hypoxanthine transport were associated with a decrease (≈ 2-fold) and an increase (≈ 4-fold), respectively, in V_{max} of transport for the two transport pathways. No significant change in K_m was detected following 24 h exposure to 16 nM PMA.

3.4. Effects of protein kinase C inhibitors on PMA-induced changes in hypoxanthine transport

The involvement of protein kinase C in the PMA-induced effects on hypoxanthine transport by LLC-PK$_1$ cells was further evaluated by examining the effects of staurosporine, a potent inhibitor of protein kinase C [23]. As shown in Fig. 3, incubation of LLC-PK$_1$ cells with 100 nM staurosporine for 24 h led to ≈ 60% inhibition of Na^+-dependent hypoxanthine transport. This result was unexpected, as control experiments established that this concentration of staurosporine had no effect on hypoxanthine transport when added simultaneous with radiolabeled substrate (13 ± 1 and 11.8 ± 2.3 for Na^+-dependent hy-

Table 3
Effect of PMA on the kinetic constants of sodium-dependent and sodium-independent hypoxanthine transport by LLC-PK$_1$ cells

	K_m (μM)		V_{max} (pmol/mg protein per min)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Na^+-dependent	Control			
	2.2 ± 0.7	0.2 ± 0.1	23 ± 4	7.3 ± 1
	PMA-treated			
	1.6 ± 0.7	0.4 ± 0.08	11 ± 2	3.1 ± 0.3
Na^+-independent	Control			
	88 ± 13	52 ± 35	153 ± 5	78 ± 14
	PMA-treated			
	115 ± 32	78 ± 8	480 ± 48	428 ± 30

Cells were incubated for 24 h at 37°C with PMA at 16 nM. The initial rate of [^{3}H]hypoxanthine influx (30 s) was then determined at 22°C in the presence of Na^+ or NMG^+ or NMG^+ plus 3 mM adenine. The Na^+-dependent component of hypoxanthine influx was calculated as the difference in uptake values between cells incubated in Na^+ and NMG^+. The Na^+-independent component was obtained from the difference in initial rates of hypoxanthine uptake in NMG^+ media in the presence and absence of 3 mM adenine [5]. The kinetic constants were determined by non-linear least-squares fit of the Michaelis-Menten equation. Results from two separate experiments are given.

poxanthine influx in the absence and presence of 100 nM staurosporine, respectively). Moreover, as an inhibitor of protein kinase C, staurosporine would be expected to elicit the opposite effect to that of PMA. However, staurosporine is not specific for protein kinase C [23], and the observed inhibition of Na^+-dependent hypoxanthine transport may reflect inhibition of another protein kinase. In contrast, staurosporine (100 nM) had no detectable effect on Na^+-independent hypoxanthine transport. Furthermore, in the presence of PMA, 100 nM staurosporine added simultaneously with PMA completely eliminated the PMA-induced stimulation of uptake. This result is consistent with the proposal that protein kinase C is important in the stimulation of Na^+-independent hypoxanthine transport by PMA.

In an attempt to overcome the problem encountered with staurosporine as regards to Na^+-dependent hypoxanthine transport, the effect of calphostin C and Ro-31-8220 was examined on the PMA-induced changes in hypoxanthine transport. These compounds are highly selective inhibitors for protein kinase C [24–26]. Table 2 shows that calphostin C, which inhibits protein kinase C in a light-dependent manner [25], partially reversed the PMA-induced inhibition of Na^+-dependent and stimulation of Na^+-independent hypoxanthine transport. In contrast, calphostin C had no significant effect on Na^+-dependent and -independent hypoxanthine uptake in non PMA-treated cells. Similarly, Ro-31-8220 reduced the PMA-induced inhibition of Na^+-dependent and stimulation of Na^+-independent hypoxanthine transport in a dose-dependent manner ($\approx$ 16 and $\approx$ 51%, respectively, at 1 μM, and $\approx$ 57 and $\approx$ 89%, respectively, at 5 μM Ro-31-8220) (Table 2). Ro-31-8220 alone had no significant effect on Na^+-dependent and -independent hypoxanthine uptake.

3.5. Effects of protein synthesis inhibitors on PMA-induced changes in hypoxanthine transport

The observed PMA-induced changes in the V_{max} of Na^+-dependent and -independent hypoxanthine transport may reflect an alteration in the number of transporter molecules expressed per LLC-PK_1 cell. This could be achieved by the de novo synthesis of protein(s) that in the case of Na^+-dependent uptake leads to a reduction in transporter number, for instance, an enzyme(s) involved in transporter degradation, and in the case of Na^+-independent uptake to an increased transporter number. To test for an involvement of de novo protein synthesis in the PMA-induced changes in hypoxanthine transport by LLC-PK_1 cells, the effects of cycloheximide, an inhibitor of translation [27], and actinomycin D, an inhibitor of transcription [28], were examined on hypoxanthine influx (Table 4). Control experiments established that 20 μM cycloheximide blocked the incorporation of [^{3}H]leucine into acid-insoluble protein of PMA-treated (16 nM for 24 h) LLC-PK_1 cells by $\approx$ 88%. Table 4 shows that in the presence of PMA, cycloheximide (20 μM added simultaneously) had no effect on the inhibition of Na^+-dependent hypoxanthine transport, whereas it almost completely blocked ($\approx$ 90%) the stimulation of Na^+-independent hypoxanthine uptake. Cycloheximide had no significant effect on Na^+-dependent and -independent hypoxanthine transport in non PMA-treated cells. Similarly, 0.032 μg/ml actinomycin D had no effect on the PMA-induced inhibition of Na^+-dependent hypoxanthine transport (data not shown), but reduced the stimulation of Na^+-independent hypoxanthine uptake by $\approx$ 74 and $\approx$ 65% in two separate experiments. Actinomycin D had no effect on hypoxanthine influx via these routes in non PMA-treated cells.

3.6. Effects of PMA on LLC-PK_1 cell proliferation

PMA has been previously reported to be a potent mitogen in LLC-PK_1 cells [29,30]. This observation raises the possibility that the PMA-induced changes in hypoxanthine transport by LLC-PK_1 cells may be the result of changes in the pattern of gene expression associated with the dedifferentiation of these cells as they are stimulated to divide. To evaluate the role of cell proliferation in the PMA-induced changes in hypoxanthine transport, the time-course of DNA synthesis in PMA-treated (16 and 1600 nM) LLC-PK_1 cells was determined by measuring the incorporation of [^{3}H]thymidine into total DNA (2 h pulse) after 8 and 24 h of incubation with PMA. In comparison to untreated cells, the incorporation of [^{3}H]thymidine into the total DNA of PMA-treated cells was reduced by about half ($\approx 54 \pm 9$ and 50 ± 8 ($n = 3$) % for 16 and 1600 nM PMA, respectively) after 8 h of incubation. In contrast, after 24 h of incubation with PMA,

Table 4
The role of protein synthesis on PMA-induced changes in hypoxanthine transport by LLC-PK_1 cells

Treatment	Hypoxanthine influx (pmol/mg protein per min)			
	Na^+-dependent	P	Na^+-independent	P
Control	9.5 ± 2.4		2.1 ± 0.4	
PMA (16 nM)	2.8 ± 0.9	S	10.5 ± 1.8	S
Cycloheximide (20 μM)	6.2 ± 2.0	NS	2.0 ± 0.36	NS
PMA (16 nM) plus Cycloheximide (20 μM)	2.5 ± 1.0	NS [a]	3.0 ± 0.1	S [a]

Cells were incubated for 24 h at 37°C with 0.08% (v/v) DMSO (control) or 16 nM PMA, or 20 μM cycloheximide, or 16 nM PMA plus 20 μM cycloheximide. Subsequently, the initial rates of Na^+-dependent and -independent hypoxanthine influx (2 μM) were determined at 22°C. Values represent the means ± S.E.M. of three separate experiments.

[a] The data set was compared with the PMA-treated cells. For the other data sets they were compared to the control.

[^{3}H]thymidine incorporation was stimulated by 4.1 ± 0.5- and 2.2 ± 0.5-fold for 16 and 1600 nM PMA, respectively, as compared with untreated cells. These results suggested that the time-course of PMA-induced cell proliferation and inhibition of Na^+-dependent hypoxanthine uptake (see Fig. 1) were different, i.e. maximal inhibition of Na^+-dependent uptake occurred after 8 h of incubation, whereas stimulation of [^{3}H]thymidine incorporation occurred after 24 h. Moreover, maximal inhibition of Na^+-dependent uptake was coincident with the inhibition of [^{3}H]thymidine incorporation observed after 8 h of incubation, as well as with the stimulation of incorporation observed after 24 h. There was, however, a correlation between PMA-induced stimulation of Na^+-independent hypoxanthine transport and cell proliferation. Both occurred maximally after 24 h of incubation with 16 nM PMA. However, 1.6 μM PMA also stimulated [^{3}H]thymidine incorporation, although to a lesser extent and without an effect on Na^+-independent hypoxanthine uptake (see Fig. 1, lower panel and Fig. 2).

4. Discussion

In the present study we have evaluated the role of protein kinase C in the regulation of nucleobase transport in LLC-PK_1 renal epithelia. The data indicate that long-term exposure of LLC-PK_1 cells to PMA led to a protein kinase C-mediated time and dose-dependent inhibition of Na^+-dependent hypoxanthine transport that occurred independently of PMA-induced effects on cell proliferation. In contrast to the PMA effect on Na^+-dependent hypoxanthine transport, the PMA-induced stimulation of Na^+-independent hypoxanthine uptake required the synthesis of new protein and correlated with an increase in LLC-PK_1 cell proliferation.

Evidence for the involvement of protein kinase C in mediating the changes in hypoxanthine transport induced by PMA includes the following. First, PKC is the only documented receptor for PMA [7,10], and activation of PKC in LLC-PK_1 cells by PMA (10–1000 nM) has been shown previously to result in the translocation of the enzyme from the cytoplasm to the plasma membrane [31] in agreement with observations made on many other cell types [7]. In the present study, the concentrations of PMA found to elicit half-maximal effects on hypoxanthine transport are close to the affinity constant for phorbol ester binding to PKC [32]. Second, the phorbol ester, 4α-phorbol, which does not activate PKC, did not mimic the effects of PMA on hypoxanthine transport. Third, the inhibitory effect of PMA on Na^+-dependent hypoxanthine transport was mimicked by adding the DAG kinase inhibitor, R59022, which will lead to an increase in endogenous DAG. However, R59022 failed to mimic the stimulatory effect of PMA on Na^+-independent hypoxanthine transport. Nevertheless, it should be noted that the concentration of PMA required to cause half-maximal inhibition of Na^+-dependent hypoxanthine transport was an order of magnitude lower than that required for half-maximal stimulation of Na^+-independent hypoxanthine transport (see Fig. 2). Thus, it is possible that the concentration of endogenous DAG was not elevated sufficiently to initiate the events that lead to stimulation of Na^+-independent hypoxanthine transport. An alternative or perhaps complimentary explanation for the lack of effect by the DAG kinase inhibitor is that the stimulation of Na^+-independent hypoxanthine transport and possibly cell proliferation that occurred at higher PMA concentrations may be due to the down-regulation of cellular PKC or of a particular isoform, and not its initial activation. Endogenous DAG is unlikely to be able to down-regulate PKC [7], and thus, it would be unable to mimic the effects of high concentrations of PMA due to down-regulation of PKC. Finally, the PMA-induced effects on hypoxanthine transport were reversed by Ro-31-8220 and calphostin C, potent and selective inhibitors of PKC.

Kinetic analysis demonstrated that both the PMA-induced inhibition of Na^+-dependent and stimulation of Na^+-independent hypoxanthine transport resulted from changes in the V_{max} of transport rather than a change in K_m. For the Na^+-independent hypoxanthine transporter, but not the Na^+-dependent hypoxanthine transport system, the change in V_{max} involved an increase de novo synthesis of either a protein activator, some component(s) of the transport system or the transporter itself. Furthermore, experiments with actinomycin D suggested that the PMA-induced synthesis of this new protein(s) involved a transcriptional control mechanism. Control experiments established that the reduction in Na^+-dependent hypoxanthine transport was not due to a reduction in the membrane Na^+ gradient (Table 1).

In an earlier study, expression of the Na^+/glucose cotransporter (SGLT1) in LLC-PK_1 cells was also found to be inhibited by PKC activation and dedifferentiation of the cells following reseeding of confluent cells at a subconfluent density [33]. These changes in Na^+-dependent glucose transport activity were the result of changes in the level of SGLT1 mRNA and SGLT1 mRNA was also undetectable in proliferating cells [33]. Preliminary data from our laboratory has found that in contrast to Na^+-dependent glucose transport, Na^+-dependent hypoxanthine transport activity remained unchanged as proliferating LLC-PK_1 cells progressed to a differentiated state (confluent cells). However, the rate of Na^+-independent hypoxanthine influx (1 μM) decreased by 67% from 1 day preconfluent to 8-day postconfluent. These results are consistent with the notion that the stimulation of Na^+-independent hypoxanthine transport by PMA treatment is a property of LLC-PK_1 cells dedifferentiating. A previous study [5] had demonstrated that the Na^+-independent hypoxanthine transporter in LLC-PK_1 cells was preferentially located at the basolateral surface of the cell. Thus, increased levels of Na^+-independent hypoxanthine transport in proliferating LLC-PK_1 cells may be vital to ensure maximal uptake of this nucle-

obase from circulating purines for the purine salvage pathway. A further physiological implication of the data is that they support an involvement of PKC in regulating renal proximal tubule cell differentiation [34].

Although it is clear from the foregoing discussion that the effects of PMA on both transport systems were due to activation of PKC, a number of observations indicated that the PMA-induced effects on the two routes of hypoxanthine entry occurred via distinct mechanisms. First, the time-courses of the effects of 16 nM PMA on hypoxanthine transport via Na^+-dependent and -independent routes were different (see Fig. 1, upper panel). Maximum inhibition of Na^+-dependent hypoxanthine transport was observed at about 8 h, whereas incubation with PMA for this period of time had no effect on Na^+-independent hypoxanthine entry. Second, as noted above, the effects on hypoxanthine entry via the two routes exhibited an order of magnitude difference in sensitivity to PMA. Finally, the PMA-induced effects on hypoxanthine entry via the two permeation pathways exhibited different sensitivities to reversal by the PKC inhibitor, Ro-31-8220. These observations taken together suggest that different PKC isoforms are probably responsible for the PMA-induced effects on hypoxanthine transport by LLC-PK_1 cells. Regulation of the Na^+-dependent nucleobase transporter seems to involve the activation of a PKC isoform that may not to be down-regulated with prolonged PMA exposure. In contrast, the longer term dedifferentiation of LLC-PK_1 cells that results in the stimulation of Na^+-independent nucleobase transport appears to require the down-regulation of a different PKC isoform. Therefore, at the present time it is uncertain whether the effects of PMA on hypoxanthine transport by LLC-PK_1 cells are due to the initial activation and/or the subsequent depletion of PKC. Future studies examining the time-course of loss of PKC activity and the subcellular localization of PKC isozymes should clarify the mechanism of PKC modulation of hypoxanthine transport.

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