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Effect of second messenger systems on oxalate uptake in renal epithelial cells

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Abstract The oxalate transport system along with protein phosphorylation appears to be deranged in stone formers. This study was undertaken to characterize in LLC-PK_1 cells in culture the effect of altering specific intracellular second messenger systems on oxalate uptake. Cellular uptake experiments were performed at 37 °C in buffer [265 mM mannitol, 5 mM NaOH, 5 mM KOH, 10 mM Ca-EGTA, 25 mM HEPES/TRIS, pH = 7.4 or in Hank's balanced salt solution (HBSS)] containing 200 μM labeled oxalate (1-^{14}C, 0.3 μCi). Cells were preincubated with DAG (final concentration of 100 μM), phorbol myristate acetate (10 μM), forskolin (50 μM), 8-bromo-cyclic AMP (50 μM), trifluoroperazine (20 μM) and low molecular weight heparin (1 mg/ml) for 10 min in the presence and absence of the anion transport inhibitor DIDS (100 μM) and the effect(s) on oxalate uptake at 10, 25 and 45 min incubation were determined. Chemicals (DAG, forskolin, TPA and 8-bromo-cAMP) which stimulate protein kinase A or C activity resulted in an increased uptake of oxalate while inhibitors of these systems (trifluoroperazine and low molecular weight heparin) resulted in decreased oxalate uptake. The results demonstrate that oxalate uptake in renal tubular cells is modulated by protein kinase C and A dependent mechanisms.

Key words Oxalate transport · Protein kinase C · Nephrolithiasis · Cell culture

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Oxalate plays a major role in the pathophysiology of calcium oxalate renal stone disease [6]. Urinary oxalic acid concentration is an important determinant of calcium oxalate crystal precipitation and the subsequent growth of kidney stones [6, 26]. To permit better management of urinary oxalate excretion, it is important to understand the dynamics of renal epithelial cell oxalate transport.

Oxalate transport has been investigated using a variety of preparations including gastrointestinal tract [14, 18], kidney [17, 20, 21, 30] and erythrocytes [1], utilizing multiple methodological approaches [1, 14, 17, 20, 21, 30]. Our laboratory has used the technique of cell culture to characterize the uptake of oxalate in the renal epithelial cell line LLC-PK_1 [31]. In this study, oxalate uptake was time, concentration, energy, temperature and pH dependent and was inhibited by the classic anion exchange inhibitors DIDS and SITS (4-Acemido-4′-isothiocyanatostilbene-2,2′-disulfonic acid). The sensitivity to SITS and DIDS indicates that uptake is likely mediated via one of the family of anion transporter proteins [16].

The present study was undertaken to examine the influence of intracellular second messenger systems on the uptake of oxalate. Previous studies have shown an effect of intracellular messengers on anion transport in various systems. In rat duodenal brush border vesicles, cAMP-dependent protein kinase (PK-A) stimulates Cl/HCO_3 exchange [12]. Protein kinase C (PK-C) has been shown to stimulate anion secretion in enterocytes [11, 13] and cAMP stimulates oxalate secretion in rabbit distal colon studied by the Ussing chamber technique [14]. Oxalate exchange in erythrocytes is modified by both PK-A and PK-C [7, 8] and by phospholipid-sensitive calcium-independent protein kinases [5]. At the level of whole kidney, only indirect evidence obtained in rabbit medullary collecting duct demonstrated a link between PK-A activity and oxalate transport [15]. In this paper we investigated the effect of substances which modulate second messenger systems on oxalate uptake in LLC-PK_1 cells in culture.

Methods

Culture conditions

LLC-PK_1 cells (American Type Culture Collection ATCC CRL 1392) were maintained as previously described [10]. Briefly, cells were grown on 35 x 10-mm tissue culture dishes until confluent. All experiments were carried out at 37 °C, under 95% air and 5% CO_2. Cells were washed twice in either buffer (1.5 ml) consisting of 265 mM mannitol, 5 mM NaOH, 5 mM KOH, 10 mM Ca-EGTA, 25 mM HEPES/TRIS, pH = 7.4 or in Hank's balanced salt solution (HBSS). Using a Ca-EGTA buffer system enabled us to set and maintain the extracellular free calcium concentration and calculate the extracellular free oxalate concentration.

Experimental protocol

Cells were preincubated for 10 min in buffer or HBSS containing experimental drugs after which time the experiment was started. Labeled oxalate (^{14}C; specific activity 103 mCi/mmol; Amersham) was used to determine cellular uptake. Cells were incubated in the presence of 200 μM oxalate for periods of 10, 25 and 45 min and then washed 5 times with ice-cold phosphate-buffered saline, and 3 ml 1 N NaOH was added to solubilize the cells. The amount of ^{14}C oxalate taken up by the cells was quantitated using liquid scintillation counting. All experiments were done in triplicate and results are expressed as total picomoles oxalate per dish (9.6 cm^2). In several experiments, nonspecific entrapment of oxalate was determined by the addition of ^{3}H-mannitol to the buffer system. Nonspecific entrapment accounted for no more than 3–5% of the total radioactive oxalate taken up by the cells.

Chemicals

DAG (1-oleoyl-2-acetyl-rac-glycerol; Sigma), a stimulator of protein kinase C, was dissolved at 25 mg/5 ml absolute ethanol and then sonicated to produce micelles; the DAG was then added to buffer to give a final concentration of 100 μM. Phorbol myristate acetate (TPA, Sigma), a PKC stimulator, was dissolved 5 mg in dimethyl sulfoxide (DMSO) to give a final stock solution concentration of 81 μM. TPA in DMSO was added to buffer to give a final concentration of 10 μM TPA. Forskolin (Calbiochem), an adenyl cyclase activator, was dissolved at 5 mg/0.6 ml DMSO and then added to 10 ml distilled H_2O to give a final stock solution of 1 mM. Forskolin solution was then added to buffer to give a final concentration of 50 μM. Theophylline (Sigma), a phosphodiesterase inhibitor, was dissolved in distilled H_2O to give a 10-mM stock solution; theophylline was added to buffer to give a 50-μM final concentration. This was used in all experiments with forskolin and 8-bromo-cAMP. 8-Bromo-cyclic AMP (Sigma), a cyclic AMP analog, was dissolved in DMSO and distilled H_2O to give a 116-μM stock solution. 8-Bromo-cAMP stock solution was added to buffer to give a final concentration of 50 μM. Trifluoroperazine (Sigma), an inhibitor of both calmodulin and protein kinase C, was dissolved in distilled H_2O to give a stock solution of 1 mM. Trifluoroperazine stock was added to buffer to give a final concentration of 20 μM. Low molecular weight heparin (Sigma) stock solution was prepared at 10 mg/ml distilled H_2O. Heparin stock was added to buffer to give a final concentration of 1 mg/ml. DIDS (4,4′ diisothiocyanotostilbene 2,2′ disulfonic acid) (Sigma), an anion exchange inhibitor, was dissolved in DMSO to give a stock solution of 100 mM. DMSO stock solution was added to buffer to give a final concentration of 100 μM. In experiments in which DMSO was used to dissolve the test substance, an equivalent amount of DMSO was added to the control sample.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Data were analyzed for significance in a factorial ANOVA for multiple groups or a two-tailed, unpaired *t*-test using Statview (Statview, Abacus Concepts, Berkeley, Calif., USA) on a Macintosh computer (Apple Computers, Cupertino, Calif., USA) and results with a $P < 0.05$ were considered statistically significant.

Results

The effect of the addition of DAG and/or forskolin on oxalate uptake is presented in Fig. 1. Both DAG and forskolin stimulated oxalate uptake and the combination of DAG and forskolin showed no further stimulation above that obtained by DAG or forskolin alone ($P > 0.05$). The interexperimental DAG stimulation effect (alone or in combination with forskolin) on oxalate uptake was from 50 to 500%; the variability is thought to be due to the effect of the unstable, micellar nature of DAG. The effect of TPA or 8-bromo-cAMP on oxalate uptake is shown in Fig. 2. Both TPA and 8-bromo-cAMP showed a modest but significant stimulation of cellular oxalate uptake. Trifluoroperazine, an inhibitor of both calmodulin and protein kinase C activity, showed no effect on oxalate uptake by itself (Fig. 3). However, when incubated with DAG or forskolin, it significantly inhibited the stimulation of oxalate uptake by these two compounds. The inhibitory effect of low molecular weight heparin on oxalate uptake compared with the anion transport inhibitor DIDS is graphed in Fig. 4. When both heparin and DIDS were present simultaneously, a further significant decrease in oxalate uptake was observed.

The effect of DIDS, DAG and 8-bromo-cAMP on oxalate uptake was also studied in HBSS buffer (Fig. 5) and the results compared with the findings in the mannitol buffer system. The results using the 45-min time point for oxalate uptake are shown in Fig. 5. Note the lower baseline uptake of oxalate using the HBSS buffer system, likely due to the high chloride concentration of the HBSS buffer compared with the mannitol buffer. Nevertheless, similar inhibition with DIDS and stimulation with DAG and 8-bromo-cAMP are seen in this system. When DIDS was incubated with DAG or 8-bromo-cAMP, no stimulation of oxalate uptake was seen, strongly suggesting that this stimulatory effect is mediated through the anion exchanger.

Discussion

LLC-PK_1 cells have been widely used to investigate kidney cell physiology. These cells, in fact, exhibit many of the functional and morphological properties of both proximal and distal tubular renal cells, including transepithelial transport of glucose [23], amino acids [29], phosphate [24], chloride/bicarbonate [10] and

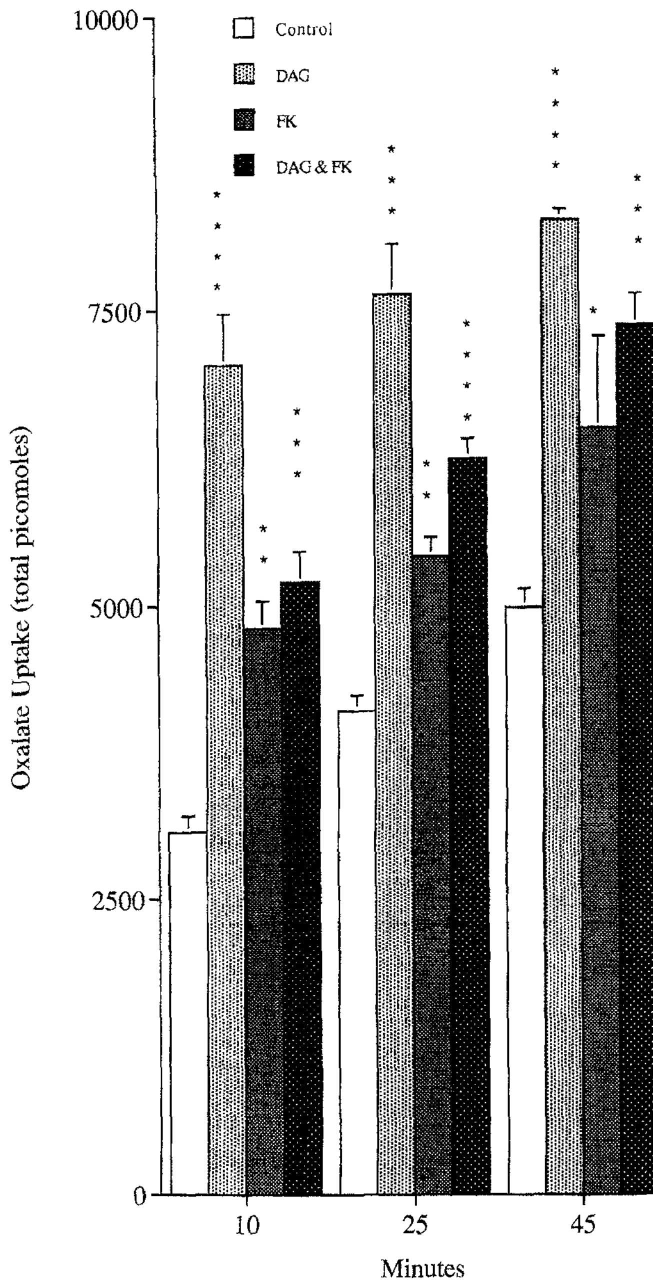

Fig. 1 Effect of DAG (100 μM) and forskolin (50 μM) on oxalate uptake at 10, 25 and 45 min. Results represent a typical experiment out of ten such experiments; results are presented as mean ± SEM of the triplicate determinations. $^{*}P < 0.03$; $^{**}P < 0.0004$; $^{***}P < 0.0002$; $^{****}P < 0.0001$ versus control

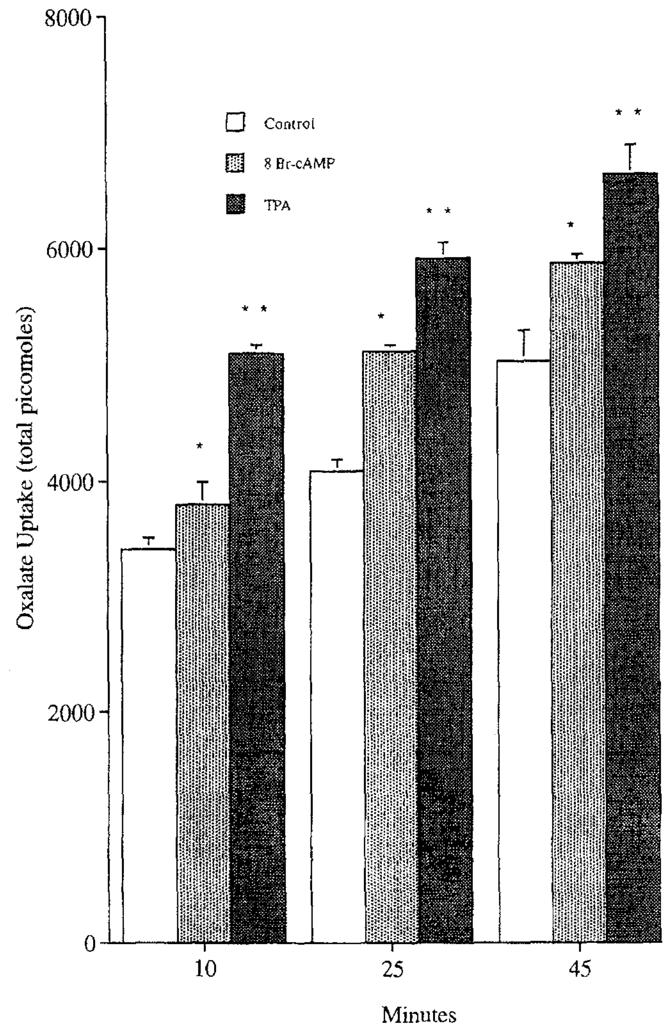

Fig. 2 Effect of 8-bromo-cAMP (50 μM) and TPA (10 μM) on oxalate uptake at 10, 25 and 45 min. The results are means ± SEM of triplicate determinations and are typical of the results obtained in five separate experiments. $^{*}P < 0.04$; $^{**}P < 0.002$ versus control

Na^+/H^+ [22]. As the site of oxalate transport appears to be the proximal tubule [9, 28], LLC-PK_1 cells represent an ideal system to define the factor(s) which control oxalate handling by the proximal renal cells. Our present study builds on our earlier studies of oxalate anion uptake in LLC-PK_1 cells [31]. From the results of the present study, it appears that the induction of cAMP synthesis by forskolin, an activator of cAMP-generating systems [27], or the stimulation of PK-C by diacylglycerol (DAG) [25], significantly increases oxalate uptake by LLC-PK_1 cells. This interpretation is further strengthened by the demonstration that 8-bromo-cyclic AMP, a cAMP analog, and phorbol 12–13 myristate acetate, which directly stimulates PKC, similarly increase oxalate uptake. Moreover, trifluoperazine (TFP), an inhibitor of both calmodulin and PK-C [32], significantly inhibits the effect of DAG on oxalate uptake and prevents the forskolin-induced increment of oxalate uptake without affecting basal oxalate uptake. These data support a role for phosphorylation of intracellular protein(s) in oxalate uptake and its control by PK-C and PK-A. Potential target proteins include band-3-related protein as well as cytoskeletal proteins such as ankyrin, spectrin and band 4.1.

Intracellular cAMP-dependent and calcium-dependent systems are intimately and multiply involved in stimulus response coupling in differentiated animal cells. Their relationships have been very well studied

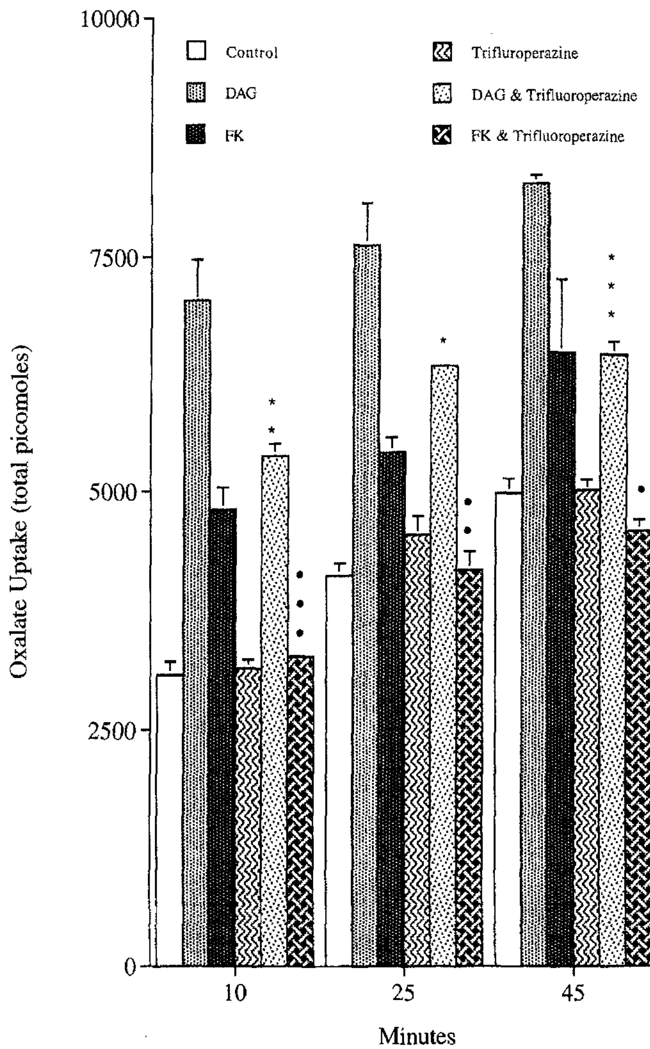

Fig. 3 Effect of trifluoroperazine (20 μM) on DAG (100 μM) and forskolin (50 μM)-stimulated oxalate uptake at 10, 25 and 45 min. The results are presented as mean ± SEM of triplicate determinations and are typical of the results obtained in five separate experiments. $^{*}P < 0.006$; $^{**}P < 0.003$; $^{***}P < 0.0001$ versus DAG. $^{\bullet}P < 0.01$; $^{\bullet\bullet}P < 0.001$; $^{\bullet\bullet\bullet}P < 0.0004$ versus forskolin

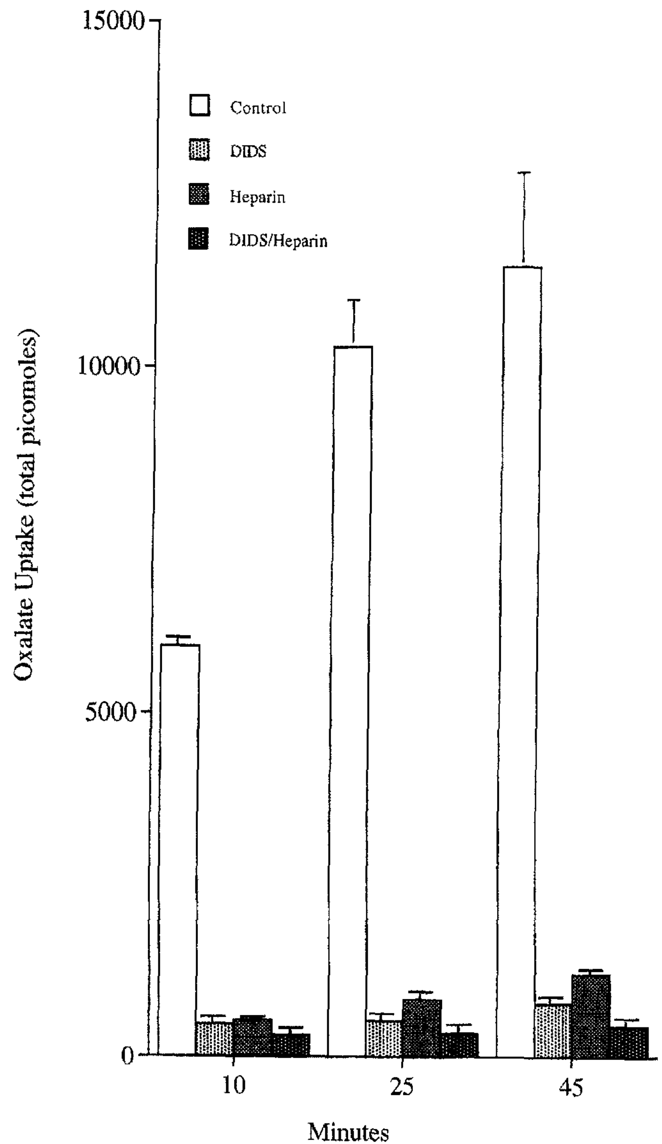

Fig. 4 Effect of heparin (1 mg/ml) and DIDS (100 μM) on oxalate uptake at 10, 25 and 45 min. The results are means ± SEM of triplicate determinations and are typical of the results obtained in five separate experiments. DIDS and heparin are significantly different ($P < 0.01$) from all other groups

[25] and at least five different patterns of synarchic regulation have been recognized. In our study, the lack of any additive effect on the rate of oxalate uptake when cAMP synthesis and PK-C activity were simultaneously stimulated favors a "redundant control" mechanism [25]. However, cAMP, besides acting on specific protein kinases, can also increase intracellular free Ca^{2+} and, therefore, could act as an indirect PK-C activator. That this may be occurring is suggested by the demonstration that the FK-induced increase of oxalate uptake is prevented by TFP, which is an inhibitor of both calmodulin and PK-C activities. Similarly, it is possible that the cAMP effect represents an indirect effect through other cAMP-mediated events.

Another important result of the present study is the data obtained using low molecular weight heparin, a glycosaminoglycan (GAG) known to be a potent inhibitor of phospholipid-sensitive, calcium-dependent protein kinase [32]. In our study, heparin alone strongly inhibits oxalate uptake and also shows an additive effect with DIDS, virtually abolishing the oxalate uptake.

The data, obtained in a physiologically relevant model system (LLC-PK_1 cells in culture), presented in this study showed changes in oxalate uptake in renal cells upon treatment with agents known to affect second messenger systems, and strongly imply that protein phosphorylation by specific kinases is involved in the control of oxalate uptake. Moreover, the results of this study raise a number of potentially clinical relevant considerations as oxalate is one of the most common constituents of urinary tract stones and plays a crucial

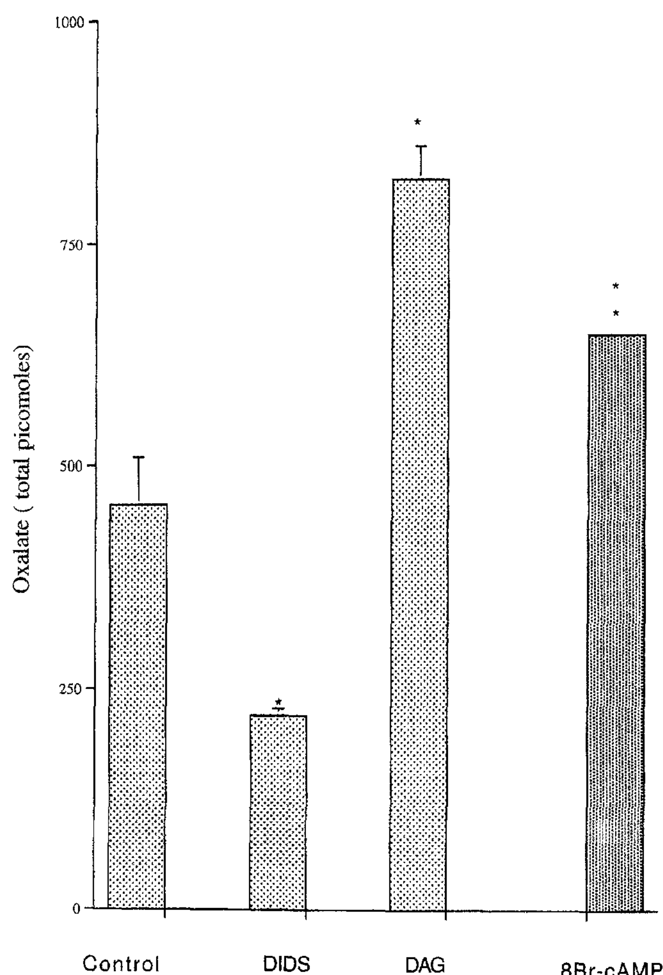

Fig. 5 Effect of HBSS buffer on DAG (100 μM) and 8-bromo-cAMP (50 μM) stimulated and DIDS (100 μM) inhibited oxalate uptake at 25 min. The results are presented as mean ± SEM of triplicate determinations and are typical of the result obtained in three separate experiments. $^{*}P < 0.001$; $^{**}P < 0.05$ versus control

role in calcium oxalate nephrolithiasis [6, 26]. Baggio and his colleagues first demonstrated the presence of cellular anomalies in calcium oxalate nephrolithiasis [1], suggesting that a cellular defect in oxalate transport is a fundamental abnormality in calcium oxalate nephrolithiasis [1, 6]. GAGs have been shown to oppose calcium oxalate crystallization in vitro [19] and there are also reports of decreased urinary excretion of GAGs in patients with renal stone disease [2, 22]. Erythrocyte GAG content, lower in stone formers than controls, correlated inversely with erythrocyte oxalate self-exchange and band 3 phosphorylation [3]. Furthermore, red blood cell oxalate self-exchange in stone formers was reduced in vitro by pretreatment with GAGs [4]. Finally, GAGs were shown to have inhibitory effects on band 3 phosphorylation and anion transport both in vitro and in vivo [3, 4]. These data support the contention that a lower erythrocyte membrane content of GAGs enhances membrane protein phosphorylation, leading to an increased rate of transmembrane oxalate flux. The present study, showing a markedly reduced in vitro cellular oxalate uptake when low molecular weight heparin is added, provides evidence for a similar role of GAGs in renal tubular cells.

In conclusion, our study demonstrates that oxalate uptake in renal tubular cells is modulated by protein kinase C and A dependent mechanisms. The demonstration that these control mechanisms for oxalate uptake are also present in renal tubular cells in culture should provide a useful system in which to further characterize the control mechanisms of oxalate uptake and to explore the pathophysiology and cellular basis of nephrolithiasis.

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