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Biochimica et Biophysica Acta 1420 (1999) 223–230

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Protein kinase C-mediated regulation of the renal Na^+/dicarboxylate cotransporter, NaDC-1

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Received 23 March 1999; received in revised form 1 June 1999; accepted 17 June 1999

Abstract

The Na^+/dicarboxylate cotransporter of the renal proximal tubule, NaDC-1, reabsorbs Krebs cycle intermediates, such as succinate and citrate, from the tubular filtrate. Although long-term regulation of this transporter by chronic metabolic acidosis and K^+ deficiency is well documented, there is no information on acute regulation of NaDC-1. In the present study, the transport of succinate in *Xenopus* oocytes expressing NaDC-1 was inhibited up to 95% by two activators of protein kinase C, phorbol 12-myristate, 13-acetate (PMA) and *sn*-1,2-dioctanoylglycerol (DOG). Activation of protein kinase A had no effect on NaDC-1 activity. The inhibition of NaDC-1 transport by PMA was dose-dependent, and could be prevented by incubation of the oocytes with staurosporine. Mutations of the two consensus protein kinase C phosphorylation sites in NaDC-1 did not affect inhibition by PMA. The inhibitory effects of PMA were partially prevented by cytochalasin D, which disrupts microfilaments and endocytosis. PMA treatment was also associated with a decrease of approximately 30% in the amount of NaDC-1 protein found on the plasma membrane. We conclude that the inhibition of NaDC-1 transport activity by PMA occurs by a combination of endocytosis and inhibition of transport activity.

Keywords: Succinate; Citrate; Sodium co-transport; Phorbol esters; Membrane trafficking

1. Introduction

The Na^+/dicarboxylate cotransporter of the renal proximal tubule, NaDC-1, reabsorbs Krebs cycle intermediates, such as succinate and citrate, from the tubular filtrate [1]. This transporter couples three sodium ions to the movement of one substrate molecule, carried as a divalent anion. One of the functions of NaDC-1 is in regulating the concentration of urinary citrate, an endogenous inhibitor of calcium stone formation [2,3]. The transport of citrate by NaDC-1 is stimulated by acidic pH [4,5]. One mechanism for this transport activation is an increase in the concentration of the preferred substrate, $citrate^{2-}$, as the pH is decreased. However, there is also evidence that chronic metabolic acidosis stimulates citrate uptake by inducing transporter activity [6], which is the result of an increase in NaDC-1 mRNA and protein [7]. Other chronic conditions, such as K^+ deficiency and starvation, also stimulate citrate transport [7–9]. The stimulation of citrate transport in chronic K^+ depletion has also been

Abbreviations: DMSO, dimethylsulfoxide; PMA, phorbol 12-myristate, 13-acetate; DOG, *sn*-1,2-dioctanoylglycerol; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate, NaDC-1, rabbit renal Na^+/dicarboxylate cotransporter

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0005-2736/99/$ – see front matter
PII: S0005-2736(99)00102-9

shown to result from increased NaDC-1 mRNA and protein abundance [10].

In contrast to the number of studies on chronic effects on NaDC-1 activity, however, there is no information on acute changes in transporter activity. Short-term regulation of membrane transporter activity can be mediated by direct modulation of the transporter protein, such as phosphorylation, or by changes in the abundance of the transporter at the plasma membrane. For example, the glutamate transporters, GLT-1 and GLAST-1, and the dopamine transporter, DAT, are directly phosphorylated in response to protein kinase C activation [11–13]. There are also examples of regulation of transporter activity by altered targeting of the proteins to or from the plasma membrane. The activities of the GABA transporter, GAT-1 [14,15], the Na^+/glucose cotransporter, SGLT1 [16], and the Na^+/H^+ exchanger, NHE3 [17] are regulated by insertion or removal processes. In the serotonin transporters, SERTs, phosphorylation and endocytosis of transporter protein are both observed when protein kinase C is activated, which implies that the phosphorylation state of the SERTs determines their trafficking in the cell [18].

In the present study, the short-term effects of protein kinases on NaDC-1 activity were investigated. NaDC-1 is acutely inhibited by activators of protein kinase C and relatively insensitive to activation of protein kinase A. Although NaDC-1 contains two consensus sequences for phosphorylation by protein kinase C, these sites do not mediate the inhibition of transport by protein kinase C. Disruption of microtubules with cytochalasin D prevents some of the effects of phorbol 12-myristate, 13-acetate (PMA). Furthermore, the activation of protein kinase C is accompanied by a 30% decrease of NaDC-1 protein at the cell surface, indicating that part of the inhibition is due to endocytosis of the transporter. The remaining reduction of NaDC-1 activity most likely occurs by direct inhibition of the transporter activity.

2. Materials and methods

2.1. Xenopus oocytes

Stage V and VI oocytes from *Xenopus laevis* were dissected and collagenase treated as described previously [19]. Oocytes were cultured at 18°C in Barth's medium supplemented with 5% heat-inactivated horse serum, 2.5 mM sodium pyruvate and 50 mg/l gentamicin [19]. Culture dishes and medium were changed daily.

2.2. In vitro cRNA transcription

The wild-type and mutant NaDC-1 cDNAs in pSPORT plasmid were used as templates for cRNA synthesis [19]. Plasmids were linearized with *Not*I and in vitro cRNA transcription was done using the T7 mMessage mMachine Kit (Ambion). The cRNAs were resuspended in water to a final concentration of 0.5–1 μg/μl.

2.3. Transport experiments

Transport of [^{14}C]succinate (DuPont-NEN) was measured 5–7 days after oocyte injections, also as described [19]. Sodium and choline buffers (in mM) were as follows: 100 NaCl or cholineCl, 2 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES-Tris, pH 7.5. The oocytes were rinsed briefly with choline buffer to remove sodium and serum. Transport was initiated by replacement of the choline rinse with 0.4 ml of the appropriate transport buffer as described in the figure legends. Transport was stopped by the addition of 4 ml ice-cold choline buffer followed by removal of extracellular radioactivity with three additional washes in cold choline buffer. Individual oocytes were transferred to scintillation vials and dissolved in 0.5 ml 10% SDS. Scintillation cocktail was added and radioactivity was counted. Counts in control uninjected oocytes were subtracted from the counts in cRNA-injected oocytes. Data are presented as means ± S.E.M. Statistical analysis was done using the SigmaStat Program (Jandel Scientific).

2.4. PMA experiments

Xenopus oocytes were preincubated at room temperature in Barth's solution containing PMA (usually 50 nM) dissolved in dimethylsulfoxide (DMSO). Control oocytes were incubated in Barth's solution containing DMSO alone (the amount of DMSO was 1 μl/ml of solution). The PMA or DMSO-containing

solution was removed with four washes of choline buffer at room temperature, after which the transport buffer was added. To determine the effects of different drugs, the oocytes were treated with the test compound before the incubation with PMA. Staurosporine or okadaic acid stock solutions were prepared in DMSO. Oocytes were preincubated in Barth's solution containing DMSO alone, or containing okadaic acid (1 μM), or staurosporine (10 and 20 μM) for 1 h at room temperature. Colchicine and cytochalasin D stock solutions were prepared in ethanol. In those experiments, oocytes were preincubated in Barth's solution containing either ethanol alone, colchicine (20 μM) for 5 h or cytochalasin D (20 μM) for 1 h at 18°C. After the first incubation with the test agents, the solutions were removed with three washes of room temperature choline buffer. The oocytes were then incubated for 30 min at room temperature in Barth's solution containing DMSO or PMA dissolved in DMSO. The solutions were again removed with three washes of choline buffer and the transport buffer containing [^{14}C]succinate was added for measurement of succinate uptakes.

2.5. Site-directed mutagenesis

The two consensus sites for protein kinase C phosphorylation in NaDC-1 [19], Thr-186 and Ser-366, were altered by site-directed mutagenesis. The mutagenesis was done using the oligonucleotide-directed method [20] with reagents from the Muta-Gene plasmid kit from Bio-Rad, according to manufacturer's directions. The sequences of the oligonucleotides used in the mutagenesis procedure were: (Mutant P1) 5′-CACCTTGCTGTTTTCCTTCTGGGG-3′ and (Mutant P2) 5′-GACTCTCCCGTTGGCGTCGGAGAA-3′. The identity of the mutants, T186N and S366N, was verified by sequencing. The mutants are designated using the single letter amino acid code followed by the number indicating the position of the residue in wild-type NaDC-1. The second letter following the sequence number indicates the amino acid replacement at that position.

2.6. Cell surface biotinylation of oocytes

The cell surface expression of NaDC-1 was tested using a membrane impermeant reagent, Sulfo-NHS-LC-Biotin (Pierce), as described [21]. For each biotinylation experiment, groups of five oocytes were rinsed three times with 4 ml ice-cold phosphate-buffered saline, pH 8 (PBS) [21]. The oocytes were then incubated 10 min at room temperature in 0.5 ml PBS containing 0.5 mg/ml Sulfo-NHS-LC-Biotin. The biotinylation reagent was removed with four washes of 4-ml ice-cold PBS. The oocytes were then dissolved in lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.6, 1% Triton X-100) for 30 min on ice. The solubilized oocytes were centrifuged 15 min at $14\,000 \times g$ and the supernatants transferred to new tubes. The biotinylated proteins were incubated with 50 μl ImmunoPure Immobilized Streptavidin beads (Pierce) for 1 h at 4°C. The biotin–streptavidin–agarose bead complexes were washed four times by suspension in lysis buffer followed by centrifugation. The final pellets were resuspended in the appropriate sample buffer and boiled 5 min before being loaded onto a polyacrylamide gel for SDS-PAGE [22].

2.7. Western blots of cell surface proteins

The transporters precipitated by the biotinylation–streptavidin precipitation procedures were identified by Western blotting as described previously [22]. Briefly, oocyte proteins were separated by SDS-PAGE and blotted onto nitrocellulose [22]. The nitrocellulose membranes were blocked for a minimum of 1 h in PBS-TM (phosphate-buffered saline containing 0.05% Tween 20 and 0.5% Carnation instant dried milk). The Western blots were probed with an antibody directed against a fusion protein of NaDC-1, which was raised in rabbits [23]. The primary antibody was diluted 1:10 000 in PBS-TM, and applied for 1 h, after which the secondary antibody, horseradish peroxidase-linked anti-rabbit Ig (Amersham), was diluted 1:10 000 in PBS-TM and applied for 1 h. All incubations and washes were done at room temperature. Antibody binding was visualized by enhanced chemiluminescence using the Supersignal CL-HRP substrate system (Pierce). Molecular weights were estimated by comparison with prestained protein standards (Bio-Rad). The Western blots were scanned with a Microtek Scanmaker E3

scanner, and peak mass and intensity were calculated using SigmaGel software (Jandel Scientific).

3. Results

The regulation of NaDC-1 activity by protein kinases was tested by measuring the effect of protein kinase activators on succinate transport in *Xenopus* oocytes expressing NaDC-1. As shown in Fig. 1, preincubation of oocytes with the protein kinase C activators, phorbol 12-myristate, 13-acetate (PMA) or *sn*-1,2-dioctanoylglycerol (DOG) [24,25], resulted in an inhibition of succinate transport by approximately 80%. In the same oocytes, stimulation of protein kinase A with 8-bromo-cAMP caused only a minor stimulation, approximately 10%, of succinate transport by NaDC-1 (Fig. 1). Treatment of oocytes with vehicle alone (0.1% DMSO) had no effect on succinate transport (not shown). The inhibitory effect of PMA on succinate transport by NaDC-1 was dose-dependent, with approximately 50% inhibition

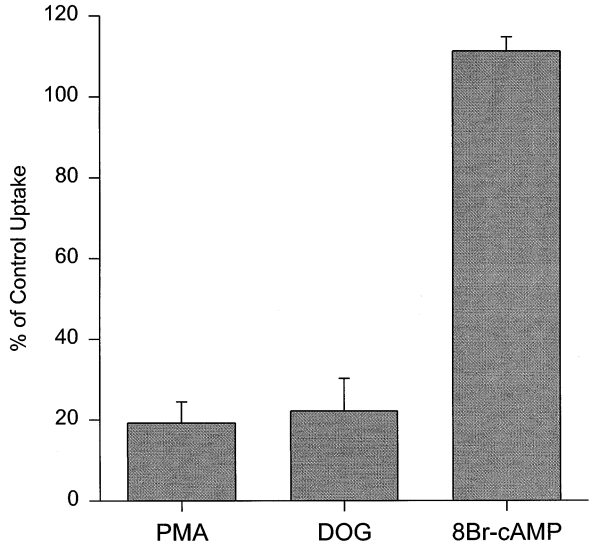

Fig. 1. Effect of protein kinase activators on succinate transport by NaDC-1. *Xenopus* oocytes expressing NaDC-1 were preincubated 30 min with Barth's solution containing DMSO (controls), 50 nM PMA, 10 μM DOG, or 2 mM 8-Br-cAMP. The preincubation buffers were washed away and transport of 100 μM succinate was measured over 30 min. Uptakes are expressed as percentage of control uptake rates (318 ± 23 pmol/oocyte/h, $n = 5$).

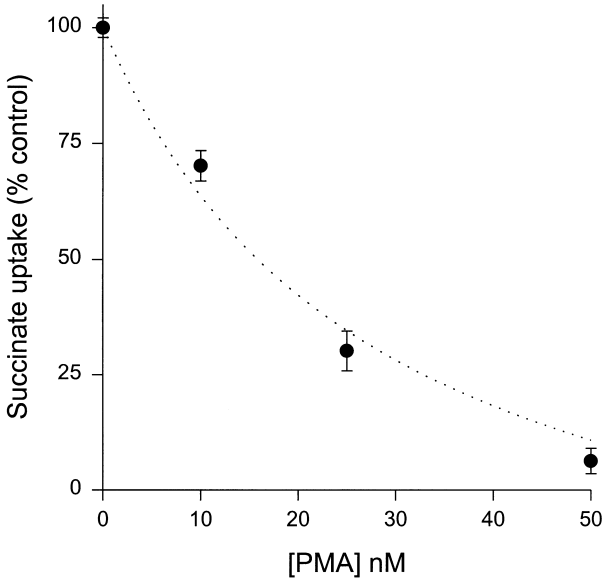

Fig. 2. Concentration dependence of inhibition by PMA. Oocytes expressing NaDC-1 were preincubated with up to 50 nM PMA, as described in Section 2. Succinate transport was measured for 30 min. The uptake rates are expressed as percentage of uptakes in control oocytes, which were incubated with DMSO only (1673 ± 35 pmol/oocyte/h, $n = 5$).

seen at 12 nM PMA (Fig. 2). The maximal inhibition was approximately 95% at 50 nM PMA (Fig. 2).

Staurosporine is a membrane-permeable, broad-specificity inhibitor of protein kinases, including protein kinase C [26]. As shown in Fig. 3, preincubation of oocytes with 10 μM staurosporine partially prevented the inhibition seen with PMA, whereas 20 μM staurosporine completely prevented the effects of PMA. Therefore, a protein kinase, most likely protein kinase C, is involved in mediating the inhibitory effect of PMA. Oocytes expressing NaDC-1 were also treated with okadaic acid before being incubated with PMA. Okadaic acid, which inhibits protein phosphatases [27], should potentiate the effects of PMA. However, okadaic acid had no effect on the response of the oocytes to PMA (Fig. 3). Okadaic acid was also ineffective in altering the response of NaDC-1 to a lower concentration of PMA (25 nM) (not shown). Both okadaic acid and staurosporine had slight inhibitory effects (5 and 14%, respectively) on succinate transport by NaDC-1 (not shown), suggesting that the endogenous activity of protein kinases in the oocytes may affect transport activity of NaDC-1.

The sequence of NaDC-1 contains two consensus

sites for phosphorylation by protein kinase C, at Thr-186 and Ser-366 [19,28]. According to the current secondary structure model of NaDC-1, Thr-186 is located in a cytoplasmic loop, between transmembrane domains 4 and 5, and Ser-366 is located in an extracellular loop, between transmembrane domains 7 and 8. To test whether the effects of PMA on succinate transport by NaDC-1 are mediated through either of these potential phosphorylation sites, each of these residues was mutated to an asparagine. Both mutant transporters exhibited succinate transport activity that was almost as high as the wild-type NaDC-1 (Fig. 4), indicating that Thr-186 and Ser-366 are not required for transport activity or protein targeting. There was no difference in PMA sensitivity between the wild-type NaDC-1 and T186N and S366N mutant transporters (Fig. 4). Therefore, the inhibitory effects of PMA on NaDC-1 are independent of phosphorylation of the protein kinase C consensus sites.

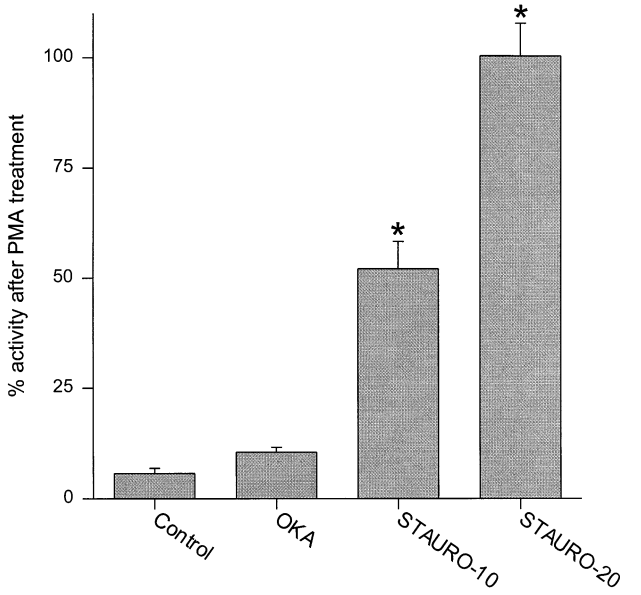

Fig. 3. The inhibitory effect of PMA can be prevented by staurosporine. *Xenopus* oocytes expressing NaDC-1 were preincubated with okadaic acid (OKA, 1 μM) or staurosporine (STAURO, 10 and 20 μM), or DMSO alone (controls) as described in Section 2. The oocytes were then incubated with or without 50 nM PMA. Finally, the transport of 100 μM succinate was measured for 30 min. The results show the succinate activity remaining after PMA treatment expressed as a percent of transport activity in the absence of PMA (mean ± S.E.M., $n = 3$–5 batches of oocytes). The asterisk (∗) indicates a significant difference, $P < 0.05$, in the staurosporine-treated groups compared with vehicle-treated controls.

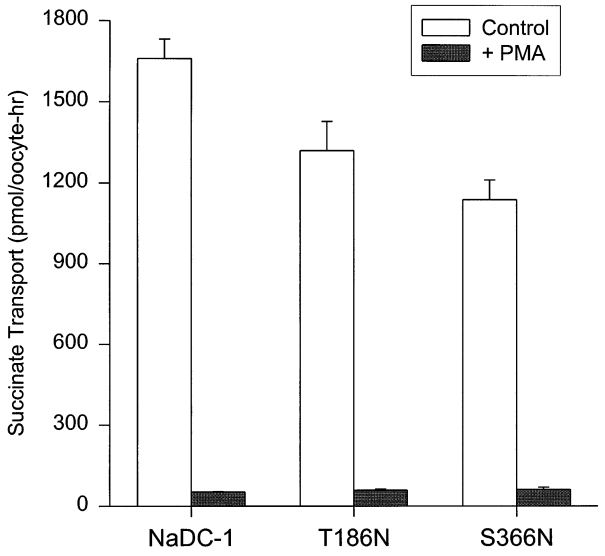

Fig. 4. Sensitivity to PMA inhibition by wild-type NaDC-1 and phosphorylation site mutants, T186N and S366N. Oocytes were preincubated 30 min in Barth's solution containing DMSO (controls) or 37.5 nM PMA. Transport of 100 μM succinate was then measured during the next 30 min. When expressed as percentage of control for each group, the activity remaining after PMA treatment was 3.2% (NaDC-1), 4.5% (T186N) and 5.4% (S366N). Data shown are means ± S.E., $n = 5$ oocytes.

One possible mechanism for the inhibition of NaDC-1 activity by PMA is by endocytosis of cell surface transporters. Two agents that affect protein trafficking through the cell were tested for their ability to prevent the effects of PMA on succinate transport (Fig. 5). Colchicine, which acts by disrupting microtubule polymerization, had no effect on inhibition of succinate transport by PMA. However, cytochalasin D, which disrupts microfilaments, partially prevented the inhibitory effect of PMA on succinate transport (Fig. 5). In four separate experiments, PMA inhibited transport to 9 ± 2% of control in the absence of cytochalasin D, but after incubations with both cytochalasin D and PMA the transport was only inhibited to 42 ± 8% of control.

The cell surface expression of NaDC-1 after treatment with PMA was monitored with a membrane impermeant derivative of biotin, sulfo-NHS-LC-biotin, followed by Western blotting of the cell-surface proteins with a specific anti-NaDC-1 antibody. There was a dose-dependent reduction in the amount of NaDC-1 protein expressed at the plasma membrane

after incubation with PMA (Fig. 6). Western blots of NaDC-1 expressed in oocytes typically have two protein bands, in this case at 76 and 55 kDa, that represent differently glycosylated forms of the transporter [22]. The total NaDC-1 protein was reduced 6% (10 nM PMA), 19% (25 nM PMA) and 32% (50 nM PMA). Both glycosylated forms of NaDC-1 were reduced by a similar amount. For example, after treatment with 50 nM PMA, the 76 kDa protein was reduced by 27% and the 55 kDa protein was reduced by 31%. Therefore, the reduction of NaDC-1 activity after PMA treatment is partly accounted for by the removal of transporter protein from the plasma membrane.

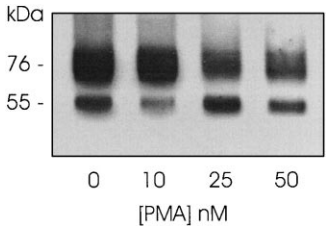

Fig. 6. Cell surface expression of NaDC-1 in control (DMSO-treated) and PMA-treated oocytes. Oocytes were treated with PMA (at 10, 25 or 50 nM) in DMSO or DMSO alone (0 PMA condition) as described in Section 2, after which the cell surface proteins were biotinylated with sulfo-NHS-LC-biotin. The proteins were then precipitated with streptavidin–agarose and run on Western blots. Blots were probed with 1:10 000 dilution of primary antibody followed by 1:10 000 dilution of secondary antibody.

4. Discussion

The Na^+/dicarboxylate cotransporter, NaDC-1, expressed in *Xenopus* oocytes is subject to regulation by activators of protein kinase C. A marked inhibition of succinate transport was seen when oocytes expressing NaDC-1 were treated with the phorbol ester, PMA, or with the diacylglycerol analog, DOG. The effects of PMA could be prevented by staurosporine, suggesting that the activation of a protein kinase, probably protein kinase C, is involved in the inhibition of NaDC-1 activity. The mechanism of inhibition of NaDC-1 appears to be a combination of a decrease in the amount of protein at the plasma membrane and an inhibitory effect on the activity of the transporter itself.

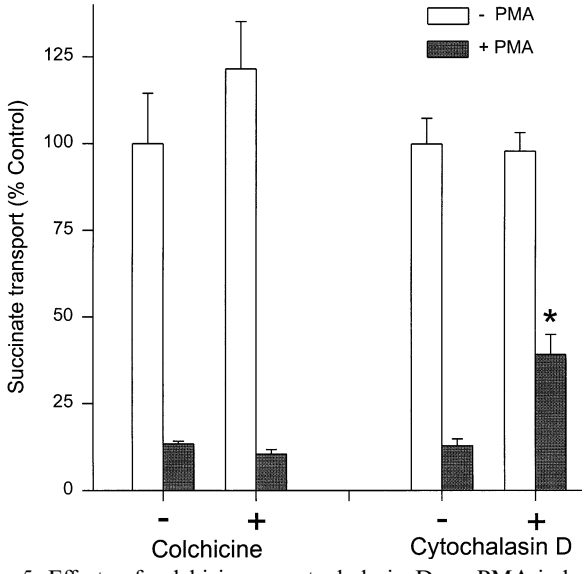

Fig. 5. Effects of colchicine or cytochalasin D on PMA-induced inhibition of NaDC-1 activity. Oocytes expressing NaDC-1 were preincubated with Barth's medium containing 20 μM colchicine or 20 μM cytochalasin D dissolved in ethanol or ethanol alone, as described in Section 2. The oocytes were then treated with 50 nM PMA dissolved in DMSO or DMSO alone. After the second preincubation, the oocytes were again washed and transport of 100 μM succinate was measured for a 15-min time period. The asterisk (*) indicates a significant difference, $P < 0.05$, between PMA treatment in the presence and absence of cytochalasin D.

Approximately one-third of the inhibition of NaDC-1 by phorbol esters can be accounted for by endocytosis of the transporter from the plasma membrane. Cell surface biotinylation of oocytes showed a maximal reduction in NaDC-1 protein by about 30% at the highest concentration of PMA. The effects of PMA on NaDC-1 activity were partially prevented by cytochalasin D, which acts by disrupting actin microfilaments. Actin microfilaments have been shown to play important roles in the endocytic pathways from apical membranes of polarized cells [29]. There was no effect of colchicine on the reduction of NaDC-1 activity by PMA. Colchicine has been shown to affect microtubule polymerization and exocytic pathways in cells, including exocytosis of the Na^+/phosphate cotransporter, NaPi-2 [30]. A decrease in the amount of protein at the plasma membrane could be due to decreased exocytosis or increased endocytosis or a combination of both. Therefore, the effect of cytochalasin D and lack of effect of colchicine suggests that the decrease in

NaDC-1 protein in the plasma membrane is likely to be mediated by increased endocytosis.

Changes in protein trafficking in response to activation of protein kinase C appear to be a common mechanism in changes in the activity of a large number of transporters. However, the proportion of transport activity affected by protein trafficking compared with direct effects on protein activity varies with specific proteins. In many transporters, all or most of the changes in transport activity can be accounted for by changes in the amount of protein in the plasma membrane. For example, in response to PKC stimulation the rabbit Na^+/glucose cotransporter SGLT1 is inhibited whereas the human hSGLT1 is stimulated, both of which are correlated with changes in transporter protein in the plasma membrane [16]. The GABA transporter, GAT-1, is stimulated by activation of PKC through increased insertion into the plasma membrane [15]. In the taurine transporter, removal of the transporter from the plasma membrane in response to activation of PKC accounts for approximately 80% of the transport inhibition [31]. However, other transporters show a combination of direct and indirect effects on protein activity in response to protein kinase C activation. The sodium–proton exchanger, NHE-3, exhibits a decrease in V_{max} after PKC activation [17]. Approximately 50% of the reduction is accounted for by endocytosis from the plasma membrane to a cytoplasmic compartment, which suggests that the other 50% of the decrease in V_{max} is likely to be due to a decrease in turnover number. Serotonin transporters are phosphorylated and rapidly internalized, in parallel with the loss of activity, in response to activation of protein kinase C [18,32]. However, it is not known whether the phosphorylation state is the signal for changes in transporter distribution or if the phosphorylated proteins show changes in activity.

Most of the inhibition of transport activity of NaDC-1 appears to be the result of a direct effect on the transporter's activity. The transporter protein is present at the plasma membrane, but the activity is reduced. One potential mechanism for modulating the activity of NaDC-1 is by phosphorylation. There are examples of transporters whose activity is directly modulated by changes in phosphorylation state. The glutamate transporter, GLT-1, is phosphorylated in response to activation of protein kinase C and transport activity is proportional to the amount of phosphorylated protein [11]. The high-affinity glutamate transporter, GLAST-1, is also inhibited by direct phosphorylation of the transporter in response to phorbol ester treatment [12]. In NaDC-1, because some of the reduction of transport activity is mediated by a reduction in protein, it is possible that the inhibition of transport activity and internalization could be related. For example, phosphorylation of the transporter could lead to inhibition of function and also serve as an internalization signal, although this remains to be tested.

The inhibition of succinate transport by PMA in NaDC-1 is not mediated through either of the protein kinase C consensus phosphorylation sequences. Mutagenesis of Thr-186 and Ser-366 to remove the potential phosphorylation sites did not alter the inhibition of transport after PMA incubation. One possible explanation is that PKC phosphorylates proteins at sites other than the consensus sites, as seen in the α1 isoform of the Na^+,K^+-ATPase [33]. Alternately, the activated protein kinase C could act on other kinases that then phosphorylate NaDC-1 or it could act on an inhibitory protein that is activated by phosphorylation. In most cases, however, the effects of PKC on transporter activity are independent of phosphorylation consensus sites, with the exception of the GABA transporter, GAT-1, which is phosphorylated at a protein kinase C consensus site [11].

The renal Na^+/dicarboxylate cotransporter exhibits adaptive changes in activity in response to chronic treatments such as metabolic acidosis, potassium deficiency (which causes an extracellular alkalosis and an intracellular acidosis), and starvation [7–9]. The transport of citrate is increased in renal brush-border membrane vesicles in response to these treatments, mediated by an increased V_{max} with no change in K_m. Preliminary experiments show that the increased activity of NaDC-1 is due to an increase in both mRNA and protein abundance, suggesting regulation of gene transcription and translation [7,10]. In this study, acute changes in protein kinase C activity caused changes in NaDC-1 activity that were independent of gene regulation or protein translation. The results suggest that protein kinase C mediated pathways are likely to be involved in rapid modulation of NaDC-1 activity.

In conclusion, the activity of the rabbit renal brush-border Na^+/dicarboxylate cotransporter, NaDC-1, expressed in *Xenopus* oocytes is very sensitive to inhibition by activators of protein kinase C, but not by activation of protein kinase A. The mechanism of this inhibition is independent of consensus sequences for protein kinase C phosphorylation. However, the inhibition occurs by a combination of microtubule-dependent endocytosis of NaDC-1 from the plasma membrane and a direct inhibition of transport activity. The results of this study suggest that NaDC-1 is likely to be regulated by acute changes in protein kinase C activity in vivo.

Acknowledgements

This study was supported by the NIH (DK46269, DK02429) and by the American Heart Association, Arizona Chapter.

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