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Protein Kinase C Regulates the Citrate Transport via Na$^+$-Coupled Citrate Transporter NaCT in HepG2 Cells

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Citrate, an intermediate of tricarboxylic acid cycle, plays a crucial role for the generation of biochemical energy and synthesis of fatty acids and cholesterol in liver. The cellular uptake of citrate is mediated by Na$^+$-coupled di- and tricarboxylate transporters, particularly NaCT. Since NaCT expression level in liver is closely related to the pathogenesis of metabolic diseases, such as non-alcoholic fatty liver disease. Therefore, it is important to elucidate the regulation mechanism of NaCT function in liver. In this study, we focused on protein kinase C (PKC), and evaluated the influence of PKC activation on the citrate transport in human hepatocellular carcinoma HepG2 cells. The uptake of citrate in HepG2 cells depended on Na$^+$, and it also occurred via a saturable process. Its Michaelis constant (K_m) and maximal velocity (V_{max}) was 5.12 mM and 106 nmol/mg protein/30 min, respectively. These results suggest that the citrate transport in HepG2 cells is primarily mediated by NaCT. In addition, we observed that the Na$^+$-dependent citrate uptake in HepG2 cells was significantly decreased by the preincubation of the cells with phorbol 12-myristate 13-acetate (PMA), a PKC activator. We also found that this decrease of citrate uptake by PMA was attributed to the reduction of V_{max}, without affecting K_m value. These results indicate that PKC regulates the transport activity of NaCT in HepG2 cells. The present findings contribute to the elucidation of the regulation mechanism of NaCT function in hepatic metabolic diseases.

Key words citrate, Na$^+$-coupled citrate transporter, HepG2 cell, protein kinase C

INTRODUCTION

Citrate is a key metabolite involved in the generation of biochemical energy via tricarboxylic acid (TCA) cycle in mitochondria.[1,2] Citrate is synthesized from acetyl coenzyme A (CoA) and oxaloacetate as an intermediate in TCA cycle, and is used as a substrate for adenosine triphosphate (ATP) production. In addition, citrate also plays important role for fatty acids and cholesterol synthesis. Citrate is a major carbon source for synthesis of fatty acids and cholesterol by being catabolized via ATP-citrate lyase, and citrate is also known to activate fatty acid synthesis. Moreover, citrate restricts glucose catabolism via inhibiting phosphofructokinase-1 in glycolytic pathway,[3] whereas citrate promotes gluconeogenesis via activating fructose-1,6-bisphosphase.[4] Thus, citrate plays a pivotal role for maintaining energy and metabolic homeostasis. Citrate is mostly metabolized in liver (~85%), and therefore it has been considered that some transport systems for citrate uptake are existed in liver.

So far, three different Na$^+$-coupled di- and tricarboxylate transporters (NaCs), NaC1/NaDC1, NaC2/NaCT, and NaC3/NaDC3, have been identified as the intracellular transport systems for intermediates in TCA cycle in mammal.[5,6] These transporters belong to *SLC13* gene family. NaDC1 (SLC13A2) is expressed in human kidney and small intestine, and transports succinate and other dicarboxylate with low affinity.[7,8] NaDC3 (SLC13A3), which is expressed more broadly including human brain, kidney, placenta, liver, and pancreas, also transports the same substrates for NaDC1 with relatively high affinity.[9,10] These two transporters also transport citrate, but their affinity tricarboxylate is lower (K_m 0.6~2 mM) than that for dicarboxylates such as succinate and α-ketoglutarate (K_m:100~500 μM for NaDC1, K_m: ~5 μM for NaDC3).[11] On the other hand, human NaCT (SLC13A5) exhibit a higher affinity for citrate, whereas it has low affinity for other intermediates in TCA cycle, such as succinate, malate, and fumarate.[12–14] NaCT is the mammalian ortholog of the *Indy* (I'm not dead yet) gene, a crtical determinant of life span in *Drosophilia melanogaster*. NaCT is a 12-transmembrane transporter, and exhibits the inward electrogenic sodium-coupled substrate transport. Since NaCT is highly expressed in human liver, citrate metabolism in liver has been considered to be predominantly regulated by NaCT.

Recently, it has been demonstrated that NaCT expression in liver is closely related to the onset and progression of metabolic diseases, such as obesity, non-alcoholic fatty liver disease (NAFLD), and type 2 diabetes, in animals and human.[15,16] Patients with obesity and NAFLD have been reported to express higher level of hepatic NaCT mRNA than healthy human.[17] Moreover, it has also been shown that the inhibition or genetic depletion of NaCT improves insulin resistance and prevents NAFLD and obesity in mice.[18] Thus, the expres-

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sion level of NaCT in liver has been recognized as a key factor for the pathogenesis of metabolic diseases, and therefore, it is important to understand how the expression of NaCT and its transport activity are regulated in liver.

It has been demonstrated that protein kinases play important roles in regulating the function of hepatic transporters via direct or indirect phosphorylation of transporters.[19–21] NaCT has also been reported to be regulated its expression by protein kinase. Nueschäfer-Rube *et al*. have been demonstrated that glucagon-dependent activation of protein kinase A (PKA) induces the expression of NaCT via cAMP-responsive element-binding protein (CREB)-dependent pathway in rats.[22] On the other hand, the effect of protein kinase C (PKC) on the activity, localization, and expression of NaCT is still unknown. Since NaCT was estimated to have one PKA phosphorylation site and four PKC phosphorylation sites, we assume that PKC would be more closely related to the transport activity of NaCT. In this study, we evaluated the transport characteristics of citrate via NaCT in HepG2 cells, which are reported to express Na^+-dependent di/tricarboxylate transporters,[26,29] and also investigated the influence of PKC activation on the citrate transport.

MATERIALS AND METHODS

Materials [^{14}C]citrate (specific activity: 116.4 mCi/mmol) was purchased from PerkinElmer (Boston, MA, USA). [^{14}C]*N*-acetyl-L-aspartate (NAA) (specific activity, 55 mCi/mmol) was purchased from American Radiolabeled Chemicals (St Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), antibiotic/antimycotic solution for tissue culture, and Sepasol-RNA I Super G were purchased from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum (FBS) was obtained from Life Technologies (Carsbad, CA, USA). 24-well cell culture plate was obtained from Corning (Corning, NY, USA). ReverTra Ace was gained from TOYOBO Co., Ltd. (Osaka, Japan). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Gö 6983 was obtained from Abcam (Tokyo, Japan). Other chemicals were all of guaranteed grade.

Cell Culture Human hepatocellular carionoma HepG2 cells were purchased from DS Pharm Biomedical Co., Ltd (Osaka, Japan). HepG2 cells were cultured in DMEM supplemented with 10% FBS and 1% anitibiotic-antimycotic at 37°C in 5% CO_2/95% air.

RT-PCR HepG2 cells were cultured in T-75 flasks (Nunc). Total RNA was isolated using Sepasol RNA I according to manufacturer's instruction. Reverse transcription was carried out with 1 μg of total RNA using ReverTra Ace, and PCR was performed according to the following conditions: 95°C for 120 s, 58°C for 45 s, 74°C for 90 s, repeated for 30 cycles. This was followed by a single additional extension step at 72°C for 7 min. The specific primers for hNaCT, hNaDC3, and GAPDH are listed in Table 1. PCR products were separated by electrophoresis in 1% agarose gel and visualized with GelRed Nucleic Acid Gel Stain (Wako Pure Chemicals).

Uptake Measurements The uptake measurements were performed at 6 days after seeding. After removal of the culture medium, the cells were washed with transport buffer. The composition of the transport buffer was 25 mM HEPES/Tris (pH 7.4), 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose. Na^+ dependent citrate transport was determined by subtracting transport values measured in the absence of Na^+ from transport values measured in the presence of Na^+. Na^+-free transport buffer was prepared by substituting NaCl with equimolar concentration of *N*-methyl-d-glucamine (NMDG) chloride. When the effect of Li^+ was investigated, Na^+-containing transport buffer was used. As a control for the Li^+ effect, an equal concentration of NMDG chloride was added to maintain the osmolarity of the transport buffer. Following incubation, the buffer containing radiolabeled compound was aspirated off and the cells were washed twice with 2 mL of ice-cold buffer. The cells were solubilized with 500 μL of 1% sodium dodecyl sulfate (SDS) in 0.2 M NaOH, and aliquots (450 μL) of samples were transferred to a counting vial, and the radioactivity associated with the cells was counted by liquid scintillation spectrometry (Model LSC6000, Beckmann, Palo Alto, CA, USA).

When the effect of PKC on Na^+-dependent citrate uptake was investigated, the cells were preincubated with PMA for 3 h prior to the uptake study. When the inhibitory effect of PKC antagonist was assessed, the cells were preincubated with Gö 6983 for 90 min prior to the study.

Data Analysis For saturation kinetics of Na^+-dependent citrate uptake, the data were fitted to the Michaelis–Menten equation:

$$v = \frac{V_{max} \times [S]}{K_m + [S]} \quad (1)$$

where V_{max} is the maximal velocity of citrate transport, K_m is the Michaelis constant, and S is the citrate concentration. To examine the single transport system of citrate uptake in HepG2 cells, Eadie-Hofstee transformation was performed:

$$v' = V_{max} - K_m \frac{v'}{[S]} \quad (2)$$

where v' is the saturable component of citrate transport.

ANOVA was used to test the statistical significance of differences between groups. Two-group comparisons were performed with Student's t test. Multiple comparisons among control groups and other groups were performed with Dunnett's test.

RESULTS

Characteristics of Citrate Transport in HepG2 Cells Prior to the transport studies, we determined the mRNA

Table 1. Primer Sequences Used in RT-PCR Reaction

cDNA	Primer sequence (5' – to - 3')	PCR Product size (bp)	Accession No.
hNaDC3[1]	forward primer: 5'-ATCGTCGGAACATCTGGAAG-3' reverse primer: 5'GGCAGGAAGATGATGATGGT-3'	793	NM_001193342
hNaCT[2]	forward primer: 5'-CGGGCTAGAGAGCAAGAAAA-3' reverse primer: 5'-GGTCATTTTGGGGTGTGAAC-3'	912	AY151833
GAPDH[3]	forward primer: 5'-CCATCACCATCTTCCAGGAG-3' reverse primer: 5'-CCTGGTTCACCACCTTCTTG-3'	576	X02231

expression of NaCT and NaDC3, which are known to express in human liver, using RT-PCR. We obtained the expression of NaCT and NaDC3 transcripts (Fig. 1). We next investigated the Na^+-dependence of citrate transport in HepG2 cells. As shown in Fig. 2A, the cellular uptake of [^{14}C]citrate was linear for up to 30 min. Thus, all uptake studies were performed with a 30-min incubation period. The involvement of Na^+ and Cl^- in the uptake process was evaluated by measuring the uptake of citrate in HepG2 in the presence and absence of Na^+ and/or Cl^-. The uptake of citrate was completely abolished when Na^+ was replaced by $NMDG^+$ in the transport buffer. Figure 2B shows the Na^+-dependent uptake of citrate into HepG2 occurred via a saturable process. The kinetic parameters were calculated by non-linear regression. The transport process of citrate was saturable with a K_m of 5.12 ± 0.72 μM and a V_{max} of 106 ± 4.91 nmol/mg protein/30 min. The Eadie-Hofstee plot (Fig. 2B, inset) was linear, providing evidence for the presence of a single transport system for citrate uptake in HepG2. However, RT-PCR revealed that not only NaCT but also NaDC3 is expressed in HepG2. This raised doubts as to whether or not citrate uptake in HepG2 is mediated by NaDC3 but not NaCT. To address this issue, we examined the effect of Li^+ on uptake of citrate and *N*-acetyl-L-aspartate (NAA), which is reported

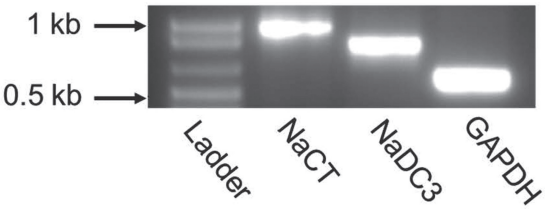

Fig. 1. mRNA Expression of NaCT and NaDC3 in HepG2 Cells

Total RNA isolated from HepG2 cells was subjected to RT-PCR using specific primers for NaCT, NaDC3, and GAPDH.

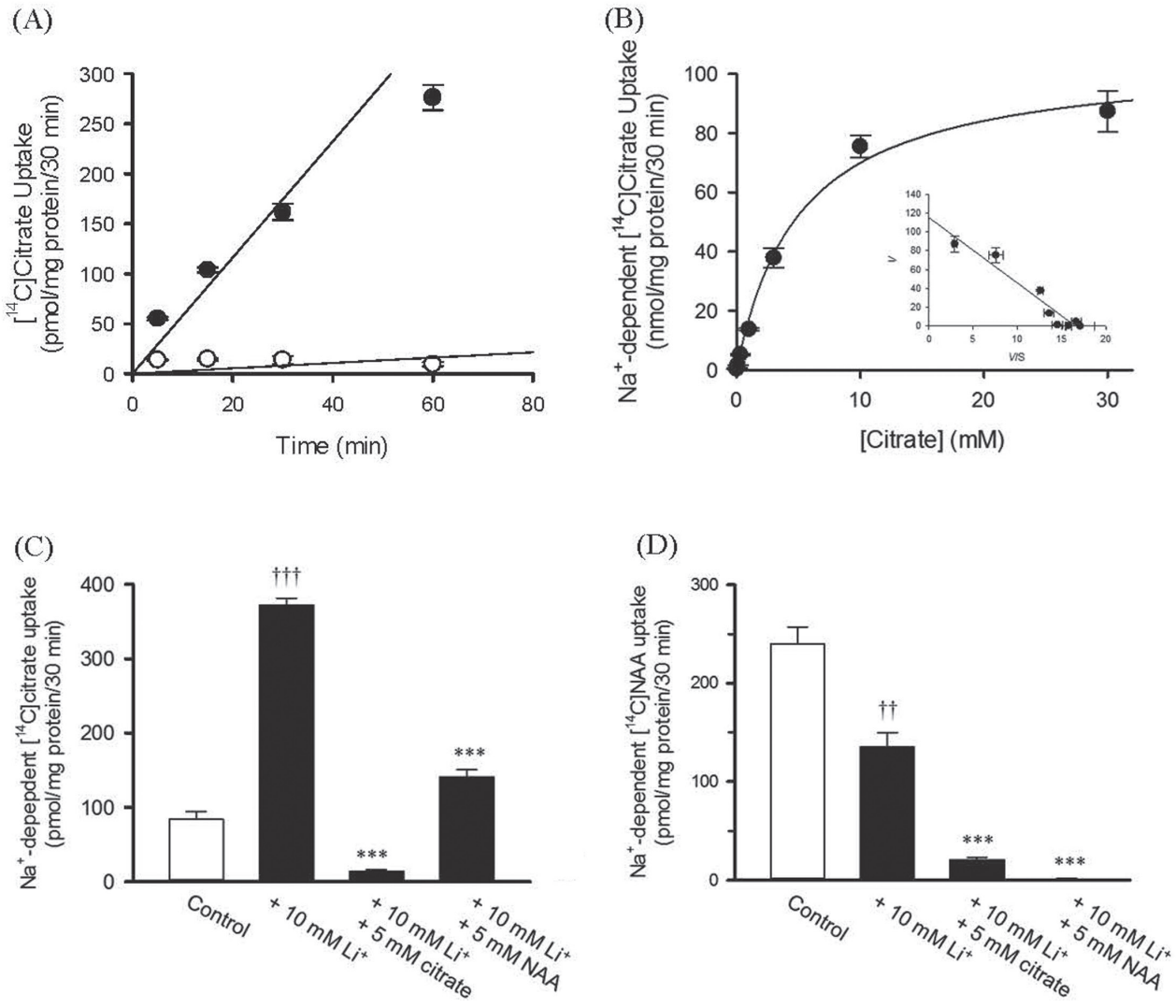

Fig. 2. Transport Characteristics of Citrate in HepG2 Cells

(A) Time course of citrate transport in HepG2 cells. [^{14}C]citrate uptake (4 μM) was measured over 60 min at 37°C in the presence (●) or absence (○) of Na^+. (B) Saturation kinetics of Na^+-dependent citrate uptake in HepG2 cells. Uptake of [^{14}C]citrate was measured in HepG2 cells during a 30-min incubation in NaCl- or NMDG chloride-containing transport buffer at pH 7.4 over a concentration range of 0.01-30 mM. Na^+-dependent uptake was obtained by subtracting uptake in the absence of Na^+ from that in the presence of Na^+. Inset: Eadie-Hofstee plot. (C & D) Effect of Li^+ on Na^+-dependent citrate (C) and NAA (D) transport in HepG2 cells. [^{14}C]citrate (4 μM) and [^{14}C]NAA (10 μM) uptake was measured for 30 min at 37°C in the presence or absence of 10 mM Li^+ and a fixed concentration of Na^+ (140 mM) in the transport buffer. The osmolarity of the transport buffer was kept by replacing LiCl with equimolar of mannitol. Each value represents the mean ± SD (n = 3). $^{††}p < 0.01$, $^{†††}p < 0.001$ compared with control; $^{***}p < 0.001$, compared with Li^+-stimulated uptake.

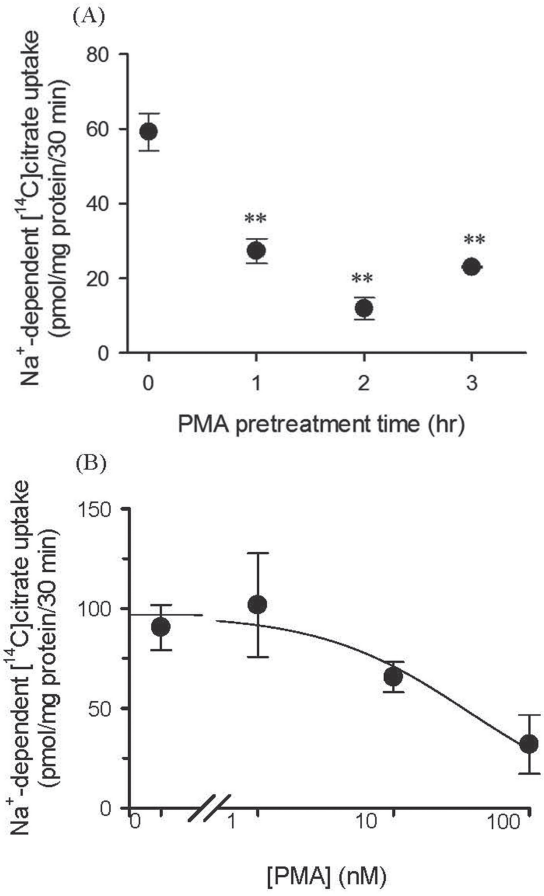

Fig. 3. Effect of PMA on Na^+-Dependent Uptake of Citrate in HepG2 Cells

HepG2 cells were preincubated with or without 100 nmol/L PMA for 1-3 h at 37°C (A) or pre-incubated over a concentration range of 1-100 nM PMA for 3 h at 37°C (B). Then, the cells were incubated with [^{14}C]citrate (8.6 μmol/L) for 30 min at 37°C. Na^+-dependent uptake was obtained by subtracting uptake in the absence of Na^+ from that in the presence of Na^+. Each value represents the mean ± SD (n = 3). **P< 0.01, compared with non-treated group.

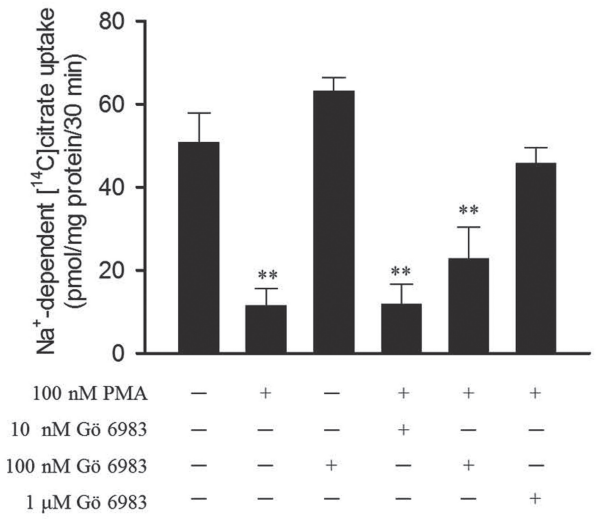

Fig. 4. Effect of Gö 6983 on PMA-Mediated Regulation of Na^+-Dependent Uptake of Citrate in HepG2 Cells

The cells were treated with or without Gö 6983 (0.01-1μM) for 30 min in culture medium prior to the PMA treatment (100 nM). Then, uptake of [^{14}C]citrate (8.6 μM) was measured during a 30-min incubation in NaCl- or NMDG chloride-containing transport buffer at pH 7.4. Na^+-dependent uptake was obtained by subtracting uptake in the absence of Na^+ from that in the presence of Na^+. Each value represents the mean ± SD (n = 3). **P< 0.01, compared with control.

to be a selective substrate for NaDC3,[10,11] in HepG2. Interestingly, human NaCT is reported to be the marked stimulation of its transport function by Li^+.[13,14] On the other hand, transport activity of NaCT derived from other species is decreased in the presence of Li^+.[12,14] Na^+-dependent citrate uptake in HepG2 was stimulated markedly by 10 mM Li^+ (Fig. 2C). On the other hand, Na^+-dependent NAA uptake was significantly reduced in the presence of 10 mM Li^+ (Fig. 2D). Therefore, Na^+-dependent citrate uptake in HepG2 might be mediated by NaCT predominantly.

Effect of PMA on Na^+-Dependent Citrate Transport in HepG2 Cells We next assessed the influences of PMA, an activator of PKC, on the transport of citrate in HepG2 cells. The Na^+-dependent uptake of [^{14}C]citrate in HepG2 cells was significantly decreased by the preincubation of the cells with PMA for more than 1 h (Fig. 3A). In addition, [^{14}C]citrate uptake in HepG2 cells was decreased in a PMA concentration-dependent manner (Fig. 3B). The concentration of PMA necessary to inhibit 50% of Na^+-dependent uptake of [^{14}C]citrate (IC_{50}) and Hill coefficient was obtained with 35.3 ± 25.6 nM and 0.80 ± 0.43, respectively.

Influence of Gö 6983 on the PMA-Induced Inhibition of Na^+-Dependent Uptake To determine the role of PKC in the inhibition of Na^+-dependent uptake caused by PMA, we studied the influence of Gö 6983, a PKC inhibitor, on the PMA-induced inhibition. As shown in Fig. 4, the inhibition of Na^+-dependent citrate uptake induced by PMA was reversed by preincubation of the cells with Gö 6983, in a ceoncentration-dependent manner. These results indicate that the activation of PKC in HepG2 cells results in the inhibition of Na^+-dependent citrate transport mediated by NaCT.

Influence of PMA on the Kinetic Parameters of Na^+-Dependent Citrate Uptake in HepG2 We next determined the effect of PMA on the kinetic parameters of Na^+-dependent citrate uptake mediated by NaCT. HepG2 cells were treated for 3 h with or without PMA (100 nM) in the culture medium prior to the saturation study. As shown in Fig. 5, PMA significantly decreased the maximal velocity (V_{max}) of Na^+-dependent citrate uptake without changing Michaelis constant (K_m). In control cells, Km for the uptake process was 5.4 ± 0.6 mM and V_{max} was 69.2 ± 2.3 nmol/mg protein/30 min. In PMA-treated cells, the K_m was 8.6 ± 2.1 mM and the V_{max} was 44.7 ± 4.0 nmol/mg protein/30 min.

DISCUSSION

Several studies revealed the PKC-dependent regulation of solute carrier (SLC) transporters function in hepatocytes.[19,21] Powell *et al*. have reported that the transport activity of organic anion transporting polypeptice 1B1 (OATP1B3) is decreased by the phosphorylation via PKC in human hepatocytes.[23] Other reports have shown that PKC activation decreases the transport activity of OATP2B1 and sodium taurocholate cotransporting polypeptide (NTCP) due to the internalization of the transporters via endocytosis.[24,25] In addition, Mayati *et al.* have demonstrated that the mRNA expression of OATP1B1, OATP1B3, OATP2B1, and organic cation transporter 1 (OCT1) was significantly decreased by the activation of PKC in human hepatoma HepaRG cells and primary human

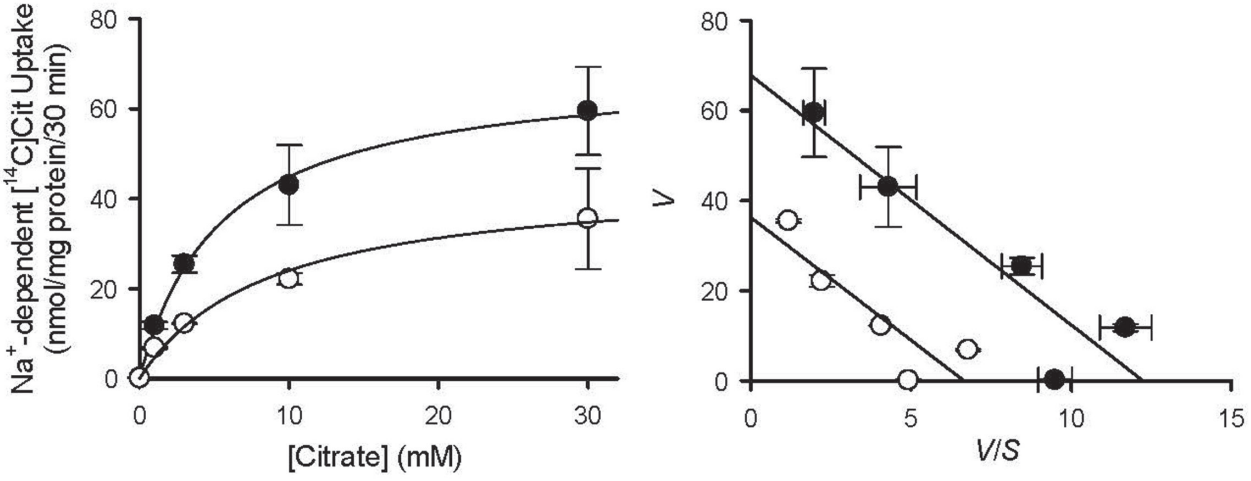

Fig. 5. Effect of PMA on Saturation Kinetics of Na^+-Dependent Uptake of Citrate in HepG2 Cells

HepG2 cells were treated with (○) or without (●) 100 nM PMA for 3 h in culture medium. After the treatment, saturation kinetic study carried out in control and in PMA-treated cells, as described in Fig. 3(B). Each value represents the mean ± SD (n = 3).

hepatocytes.[19] Thus, PKC is closely related to the transport property of some SLC transporters in liver. In this study, we describe the influence of PKC activation on the citrate transport via NaCT in HepG2 cells.

The transport process mediated by NaCT has been reported to be electrogenic, and its Na^+/substrate stoichiometry is 4: 1.[14] In the present study, we observed that the uptake of citrate in HepG2 cells depended on the presence of Na^+, and it occurred via a saturable process (Figs. 2A and B). These results suggest that the citrate transport in HepG2 cells is mediated by NaCT. Although the K_m value for citrate transport in HepG2 cells determined in the present study is higher than that in hNaCT expressed in HRPE cells (5.12 ± 0.72 mM *vs* 604 ± 73 μM),[13] the present K_m value is in good accordance with the previously reported K_m value of NaCT in HepG2 cells (5.1 ± 0.5 mM).[26] The reason for this difference are not known, but Ganapathy's group suggested that posttranslational modifications may play a role.[26] In general, in heterologous expression system in mammalian cells, transport activity was measured within 12-15 hr after transfection. There may not be sufficient time for posttranslational modifications of the newly synthesized transporter protein under these conditions. In contrast, the transporter in HepG2 cells was expressed constitutively, and therefore posttranslational modifications of the transporter protein are likely to occur these conditions.[26] On the other hand, RT-PCR revealed that NaDC3 mRNA is expressed in HepG2 cells (Fig. 1). Therefore, we determined NaDC3-mediated transport activity in HepG2 using NAA which is reported to be a selective substrate.[10,11] Although Na^+-dependent NAA uptake was observed in HepG2 cells, the uptake was decreased in the presence of 10 mM Li^+ (Fig. 2C). On the contrary, Na^+-dependent citrate uptake was remarkably stimulated in the presence of 10 mM Li^+. These results suggest that not only NaCT but also NaDC3 is functionally expressed in HepG2 cell. However, it has been demonstrated that NaDC3 can transport citrate as well as NaCT but its efficacy is low because NaDC3 recognizes only the dicarboxylate form of citrate.[13] Since citrate exists as a tricarboxylate under physiological condition (pH 7.4), the transport of citrate in HepG2 cells would be primarily mediated by NaCT.

We also found that the Na^+-dependent citrate transport in HepG2 cells was significantly decrease by the preincubation of the cells with PMA in PMA concentration- and preincubation time-dependent manner (Fig. 3). This decrease was much inhibited by Gö 6983 (Fig. 4). These results indicate that PKC regulates the transport of citrate via NaCT in HepG2 cells. It has been reported that human hepatocytes express several PKC isoforms, including classical PKC (PKC-α), novel PKC (PKC-δ, PKC-ε, and PKC-η), and atypical PKC (PKC-ζ and PKC-ι).[19] Although Gö 6983 is a pan-PKC inhibitor, PMA activates only classical and novel PKCs, not atypical PKCs, and PMA has more potential to activate PKC-η compared with PKC-α and PKC-δ.[27] Therefore, classical and novel PKCs, particularly PKC-η, would be closely related to the downregulation of NaCT-mediated citrate transport. In addition, we also reveal that the decrease of citrate transport by PKC activation in HepG2 cells is attributed to the reduction of the V_{max} (Fig. 5). Similar to the present results, several reports have reported the PKC-mediated downregulation of the transport properties of NaCs. Pajor *et al*. have demonstrated that the transport of succinate via NaDC1 is inhibited by PMA, and this occurs by the internalization of NaDC1 via endocytosis.[28] Srisawang *et al*. have also reported that the decrease of succinate transport via NaDC3 in HepG2 cells is attributed to the increased endocytosis.[29] The PKC-triggered internalization of transporters has been reported to be mediated via clathrin-dependent endocytosis, and the internalized transporters are degraded in lysosome.[24,30] Taking these findings into consideration, it is conceivable that the reduction of V_{max} of NaCT by PKC activation may occur by the decrease of NaCT protein on the cell membrane via endocytosis, followed by the degradation in lysosomes. On the other hand, there is a possibility that PKC directly inhibits the transport activity of NaCT. There are several reports demonstrating the direct inhibition of the activity of transporters by PKC via phosphorylation or other mechanisms.[23,31] Further studies are needed to identify the influence of the phosphorylation of NaCT by PKC on the transport activity of NaCT in HepG2 cells.

Recent studies have demonstrated that hepatic lipid accumulation leads to the activation of PKC in animal model of NAFLD, resulting in the NAFLD-associated hepatic insulin resistance and type 2 diabetes.[32,33] Based on these findings,

high expression of NaCT in patients with NAFLD and obesity may be caused by the activation of PKC, and therefore, it is expected that the elucidation of the relationship between PKC and NaCT in hepatic metabolic diseases leads to the development of potential treatment strategies for these diseases.

In conclusion, we demonstrated that the NaCT-mediated citrate transport in HepG2 cells was regulated by PKC. In addition, we also observed that the decrease of the cellular uptake of citrate by PMA was attributed to the reduction of V_{max}. These findings make a valuable contribution towards the elucidation of the regulation mechanism of NaCT function in hepatic metabolic diseases.

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Conflict of interest The authors declare no conflict of interest.

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