Impaired trafficking of choline transporter-like protein-1 at plasma membrane and inhibition of choline transport in THP-1 monocyte-derived macrophages

Morgan D. Fullerton, Laura Wagner, Zongfei Yuan, and Marica Bakovic

Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada

Submitted 27 May 2005; accepted in final form 28 November 2005

Fullerton, Morgan D., Laura Wagner, Zongfei Yuan, and Marica Bakovic. Impaired trafficking of choline transporter-like protein-1 at plasma membrane and inhibition of choline transport in THP-1 monocyte-derived macrophages. Am J Physiol Cell Physiol 290: C1230–C1238, 2006. First published November 30, 2005; doi:10.1152/ajpcell.00255.2005.—The present study investigates choline transport processes and regulation of choline transporter-like protein-1 (CTL1) in human THP-1 monocyctic cells and phorbol myristate 13-acetate (PMA)-differentiated macrophages. Choline uptake is saturable and therefore protein-mediated in both cell types, but its transport characteristics change soon after treatments with PMA. The maximal rate of choline uptake intrinsic to monocytic cells is greatly diminished in differentiated macrophages as demonstrated by alterations in V_{max} values from 1.973 ± 118 to 380 ± 18 nmol·mg^{-1}·min^{-1}, when the binding affinity did not change significantly (K_m values 56 ± 8 and 53 ± 6 μM, respectively). Treatments with hemicholinium-3 effectively inhibit most of the choline uptake, establishing that a choline-specific transport protein rather than a general transporter is responsible for the observed kinetic parameters. mRNAscreening for the expression of various transporters reveals that CTL1 is the most plausible candidate that possesses the described kinetic and inhibitory properties. Fluorescence-activated cell sorting analyses at various times after PMA treatments further demonstrate that the disappearance of CTL1 protein from the cell surface follows the same trend as the reduction in choline uptake. Importantly, the loss of functional CTL1 protein from the cell surface occurs without significant changes in total CTL1 protein or its mRNA level indicating that an impaired CTL1 trafficking is the key contributing factor to the reduced choline uptake, subsequent to the PMA-induced THP-1 differentiation to macrophages.

CHOLINE IS A POSITIVELY CHARGED quaternary amine, which is actively supplied for the synthesis of the major phospholipid phosphatidylcholine (58), the neurotransmitter ACh, and for the contribution of methyl groups to the one-carbon pool (17, 51). Choline deficiency has proven to be detrimental to prenatal development (59) and proven to contribute to liver dysfunction (51, 53, 60). Irregular choline transport and metabolism have also been implicated in a wide array of neurological disorders (4, 29, 30, 47).

Although several choline transport systems have been identified (15, 51), the first highly specific choline transporter, designated CHT1, has only recently been cloned in Caenorhabditis elegans (38), rat (38), mouse (1), and human (2, 37) cells. This transporter is principally responsible for supplying choline for ACh synthesis in cholinergic neurons (27), although it has also been detected in leukocytes and other nonneuronal cells (9, 42). Inhibition by hemicholinium (HC-3) and Na⁺ dependence are the main hallmarks of this neuronal, highly specific choline transporter (27). Low-affinity choline transports are broadly detected, and it is believed that they primarily supply choline for phospholipid synthesis (27). Typical representatives are the organic-cation transporters, such as OCT-1 and OCT-2, that transport various cations but could also nonspecifically transport choline (49) in the liver (13), kidney (19), and lungs (27, 33, 34).

Importantly, a distinct group of choline transporters unrelated to the OCT family, sensitive to inhibition by HC-3, named choline transporter-like proteins 1 (CTL1) have been cloned and characterized in Torpedo (40), rat (40), mouse (57), and human (55). The function of CTL1 as a choline transporter has been questioned after transfection of a Torpedo CTL1 in a yeast mutant hunt (62); however, it has recently been reaffirmed by studies of rat CTL1 in neuroblastoma cells (50). Rat CTL1 is selectively expressed in the brain, spinal cord, and colon (31, 47). Recently, we (57) cloned and functionally expressed a mouse CTL1 homologue and demonstrated that it is a choline-specific transporter sensitive to inhibition by HC-3 similarly to the rat CTL1; however, it is predominantly expressed in the mouse skeletal muscle. A human CTL1 homologue was independently cloned and characterized as a cell-surface antigen CDw92 in monocyte-derived dendritic cells, and it is also expressed in neutrophils, myeloid, and lymphoid cell lineages, suggesting its role in the immune cell function (55). This study is first to investigate the regulation of human CTL1 and its participation in choline transport in monocytes and monocyte-derived macrophages.

The general mechanisms by which choline transport is regulated remain poorly understood. However, some transporters, such as those in the Limulus brain, are known to be sensitive to inhibition of protein kinase C (PKC) by phorbol esters and a diminished uptake is associated with a decrease in HC-3 binding, suggesting a decrease in the number of transporter binding sites at the cell surface (8). It is also well known that treatments with phorbol esters induce THP-1 monocyte differentiation to macrophages (3, 24, 36). We utilize this dual role of phorbol esters to investigate the functional relationship between choline uptake and the expression of choline transporter genes, particularly CTL1. We demonstrate for the first time that CTL1 is the predominant choline transporter in THP-1 cells and that choline uptake is high in monocyctic cells and severally reduced after phorbol myristate 13-acetate (PMA) treatments as a result of diminished CTL1 protein trafficking to the plasma membrane.

Address for reprint requests and other correspondence: M. Bakovic, Animal Science and Nutrition Bldg., Rm. 346, Univ. of Guelph, Guelph, Ontario, Canada N1G 2W1 (e-mail: mbakovic@uoguelph.ca).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
**EXPERIMENTAL PROCEDURES**

PMA treatments and choline uptake. The human monocytic THP-1 cells (American Type Culture Collection) were cultured in RPMI 1640 medium (Sigma) supplemented with 1.5 g/l sodium bicarbonate and 10% fetal bovine serum (Invitrogen) in a fully humidified atmosphere of 5% CO2 at 37°C. Before analysis, cells were split to 5.4 x 106 cells/60 mm dish and either untreated or treated with 160 nM PMA (Sigma) for 15 min, 30 min, and 24 and 48 h. After treatments with PMA, THP-1 cells adhere to the dish, as the first indication of differentiation to macrophages (44, 46). Before choline uptake was measured, cells were preincubated for 1–2 h at 37°C in Krebs-Ringer-HEPES buffer (130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 10 mM HEPES, pH 7.4, and 10 mM glucose) and rinsed once in KRH buffer to remove any exogenous choline. Cells treated with PMA at different time points were then incubated with 5 nM methyl [3H]choline (83.0 Ci/mmol, Amersham Biosciences) for 5 min, followed by three rapid washes in 1 ml ice-cold KRH buffer containing 1 mM “cold” choline to stop the uptake and remove the radiolabeled choline. For choline measurement of untreated THP-1 monocytes, cells were in suspension and centrifuged at 6,000 rpm for 2 min between each step, whereas radiolabeled cells were lysed in 500 μl of an ice-cold homogenization buffer (10 mM Tris·HCl, pH 7.4, 1 mM EDTA, and 10 mM NaF) containing a 1:13 dilution of protease inhibitor cocktail (Sigma) and a 1:50 dilution of phosphatase inhibitor cocktail II (Sigma) to prevent protein degradation/dephosphorylation, followed by two freeze-thaw cycles in liquid nitrogen. The lysates were analyzed for radioactivity by scintillation counting, and the rate of choline uptake was calculated as fmol choline·mg protein−1·min−1. Protein was quantified using a BCA protein assay (Pierce).

Kinetics of choline transport. Kinetic analyses were performed as we (57) previously described (57). Briefly, THP-1 monocytes and 24-h PMA-derived macrophages were incubated with 5 nM [3H]choline for 10 min, followed by treatments with increasing concentrations of unlabeled choline (0–200 μM). Each solution contained the same amount of radiolabeled choline, which represented the total radioactivity (A, in dpm). The increasing unlabeled choline concentrations were termed B (in μM). The total choline counts in cell lysate were termed C (in dpm). The rate of choline uptake was determined as a fraction of total radioactivity measured in cell lysates and calculated using the formula (B × C)/(A × T), where T represents uptake time, in minutes. The final rate of choline uptake was expressed as nmol/min/mg. The choline uptake was plotted as a function of cold choline concentration and fitted into a saturation curve using the GraphPad Prism software. The values of Vmax and Km were determined for both untreated monocytes and PMA-derived macrophages.

mRNA expression of putative choline transporters. THP-1 cells were grown in 100-mm dishes and treated with PMA for different time points as described above. Total RNA was isolated using TRIzol reagent (Invitrogen) and reverse transcribed with the use of a poly-dT primer and Superscript II reverse transcriptase (Life Technologies). CTL1 mRNA was characterized as total CTL1 mRNA. PCR cycling conditions were 94°C for 3 min, 35 cycles of 45 s at 94°C, 30 s at 53°C, and 1 min 30 s at 72°C, and a final extension for 10 min at 72°C. The CHT1 mRNA in THP-1 cells was compared with human brain cDNA (BioChain) as a positive control using PCR conditions of 94°C for 10 min, 40 cycles of 45 s at 94°C, 45 s at 50°C, and 45 s at 72°C, and a final extension for 10 min at 72°C. For both OCT-1 and OCT-2 conditions, a human kidney cDNA (BD Biosciences) served as a positive control. OCT-1 PCR was performed under similar cycling conditions to CHT1 with only 35 cycles, whereas OCT-2 PCR used similar conditions to CTL1, with an annealing temperature of 55°C. All reactions were standardized by amplifying the β-actin mRNA under identical cycling conditions to CTL1 above. A summary of specific primers used is shown in Table 1.

Identification of type of transport by competition assays. Inhibitor HC-3 was used to block choline-specific transport in THP-1 cells. HC-3 concentrations used were 1 μM (Sigma) to block only high-affinity uptake and 200 μM to block total HC-3-sensitive uptake (38). To block all transport mediated by the nonspecific OCT family of transporters, cells were treated with 40 μM of 1-methyl-4-phenylpyridinium iodide (MPP+) or 50 μM quinine (both from Sigma) (60). The potent OCT-2 inhibitor, corticosterone (Sigma) was used to selectively block transport at 400 nM (32, 60). Undifferentiated THP-1 cells were grown as described for 60-mm dishes and kinetic measurements performed as described for choline uptake. HC-3 and corticosterone were added to the preincubated buffer in their respective tests. The MPP+ and quinine were not preincubated because less potent inhibitors were known to trans-stimulate and thus increase OCT transporter activity (14). These inhibitors were added directly with the [3H]choline solutions before testing their effect on choline uptake. The inhibition of choline transport was also measured in differentiated macrophages (48 h) with 25 μM HC-3 and 40 μM MPP+ by following the same procedure.

Western blot analysis. Total cell lysates were harvested with a lysis buffer (Promega) as we described previously (57) of untreated and PMA-treated THP-1 cells, denatured, and resolved on a 10% SDS-PAGE under reducing conditions. Resolved proteins were transferred to a PVDF membrane (Roche) and blocked in 5% skim milk in TBS-T (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20, pH 7.5) for 1 h at room temperature. The membranes were probed with the monoclonal CTL1 antibody VIM15 (Research Diagnostics) for 2 h at room temperature, washed with TBS-T and then incubated with a horseradish peroxidase-conjugated secondary goat anti-mouse IgG antibody (Promega) at a 1:10,000 dilution in milk for 2 h. Proteins were visualized using a chemilumininescent substrate (Sigma).

To estimate the ratio of plasma membrane CTL1 relative to total cell CTL1, membrane fractions of PMA-treated and untreated cells

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (Forward/Reverse)</th>
<th>Specificity</th>
<th>Product, bp</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL1</td>
<td>5'-GAGAATGGAAGCCGCTGAA-3'</td>
<td>Human</td>
<td>320</td>
<td>AJ245620</td>
</tr>
<tr>
<td></td>
<td>5'-TGAGTTCTGCTGCTGACGCC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-ATCCACCATCCATGACTT-3'</td>
<td>Human</td>
<td>168</td>
<td>AB043997</td>
</tr>
<tr>
<td>OCT1</td>
<td>5'-CCGGAGCCTTCCTCTTCC-3'</td>
<td>Mouse/human</td>
<td>211</td>
<td>U77086</td>
</tr>
<tr>
<td>OCT2</td>
<td>5'-CTGTCGACCTTTCCGATC-3'</td>
<td>Human</td>
<td>200</td>
<td>BC039899</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-AGGACGAAAGGCTCTTCGATC-3'</td>
<td>Mouse/human</td>
<td>219</td>
<td>BC002409</td>
</tr>
</tbody>
</table>

Table 1. Primer information for selected human choline transporter genes

CTL1, choline transporter-like protein-1; CHT1, choline transporter-1; OCT, organic-cation transporters.
were collected as described by Gingis-Velitski et al. (12), using an identical lysis buffer, except the buffer contained a milder detergent mixture of 1% Nonidet P-40 + 0.5% deoxycholate instead of Triton X-100. Nuclei and cell debris were removed by centrifugation at 500 g for 5 min at 4°C. Crude membrane fractions were separated by centrifugation of the supernatant at 3,000 g for 10 min and then used for the protein analysis by Western blots as described above.

Immunoprecipitation. To test the above treatments on an immunoprecipitated, natural CTL1 protein, and under non-denaturing conditions, total proteins of PMA-treated and untreated cells (500–600 µg) were precleared with 50 µl of protein G agarose beads (Sigma) for 2 h at 4°C and then centrifuged at high speed for 10 min. The precleared supernatant was incubated with the monoclonal CTL1 antibody VIM15 at a dilution of 1:50 for 2 h, and then 50 µl of protein G agarose beads were added and incubated for an additional 2 h at 4°C. Samples were briefly centrifuged, and the beads were washed four times with TBS-T for 30 min each. The beads were then diluted with 50 µl of 1 X Laemmli buffer, heated at 95°C for 5 min, and the supernatant collected after a brief centrifugation. The precipitated CTL1 was analyzed by Western blot as described above; however, both the CTL1 antibody and a monoclonal phosphoserine antibody (Sigma) were used at a dilution of 1:1,000.

Okadaic acid treatments. Cells were grown and treated with PMA as described above. Undifferentiated monocytes as well as PMA-treated macrophages were subsequently treated with 10 µM okadaic acid (Sigma) for 1 h. Therefore, the total incubation time for PMA-treated cells with or without okadaic acid was 75 min, 90 min, and 13 h. Choline uptake was then measured as described above.

Fluorescence-activated cell-sorting assays. Differentiated and undifferentiated cells (5.4 X 106 cells/60 mm dish) were washed in PBS buffer (160 mM NaCl, 3 mM KCl, 20 mM Na2PO4, and 3 mM KH2PO4) and differentiated cells detached by being gently pipetted. Cells were incubated for 15 min at room temperature in 2% goat serum in 200 µl of PBS. Negative controls were simultaneously incubated in 200 µl of PBS for 30 min. All cells were washed twice in PBS and incubated in 1:50 dilution of FITC-conjugated goat anti-mouse IgG (Sigma) in 200 µl of PBS in the dark for 30 min. Cells were washed twice more in PBS and analyzed using a fluorescence-activated cell-sorting flow cytometer (FACSCalibur; Becton-Dickinson). Cells (5 X 104) were counted per trial, and the amount of CTL1 protein surface expression was determined after the isotype control (background) fluorescence was subtracted from the anti-CTL1 antibody-treated fluorescence. The mean fluorescence of the cell population was used as a measure of the CTL1 protein levels at the cell surface.

Statistical analysis. All data are expressed as means ± SE. The analysis for the inhibition of choline uptake was a one-way ANOVA and Tukey’s test for significance, where P < 0.05 was considered significant. For the time dependencies (15 min, 30 min, 24, and 48 h PMA treatments) of both the choline uptake and the FACS CTL1 analysis, the means of individual time points were compared relative to time 0 (untreated cells) using Tukey’s multiple-comparison test at P < 0.001 and P < 0.05. Data for the choline uptake vs. [choline] were fitted into the Michaelis-Menten hyperbola. The time courses of choline uptake and CTL1 protein disappearance from the plasma membrane were compared after fitting into single-exponential decay equations. GraphPad Prism software was used for all types of data acquisition and analysis.

RESULTS

Characteristics of choline transport. Initially, we have established proper conditions for measuring the rate of choline transport (57) when an optimal uptake remains within a linear range after using 5 nM methyl [3H]choline labeling for 5 min at room temperature. It is known that THP-1 cells respond to PMA with an almost complete arrest in proliferation, become adherent, and develop a macrophage phenotype manifested with an enhanced phagocytic activity and surface expression of CD11b and CD14 antigens (46). Saturation kinetics was used to assess the type of choline transport in monocytes and differentiated macrophages. The observed rates of choline uptake were plotted against the cold choline concentration and fitted into the Michaelis-Menten equation for saturation kinetics (Fig. 1A). An excellent curve fit was obtained for both cell types with values for Km of 56 ± 8 and 53 ± 6 µM, and Vmax was determined at 1,973 ± 118 and 380 ± 18 nmol·mg−1·min−1 for monocyte and macrophage cells, respectively. Similar kinetic values were obtained after linearization of the Michaelis-Menten equation (data not shown). The nature of the saturation data

![Figure 1](http://ajpcell.physiology.org/)
demonstrate that choline transport is protein mediated in both cell types with no alterations in the binding affinity ($K_m$), suggesting that the same protein is involved in choline transport in both cell types. The transport velocity ($V_{max}$) however, is 5.2 times lower in macrophages relative to monocytes, which is most likely a consequence of different number of the binding sites (protein expression at the cell surface) in those two cells.

To establish the time course of the changes caused by the PMA treatments, THP-1 monocytes were untreated (time 0) or treated with PMA for various periods of time, i.e., 15 min, 30 min, 24 and 48 h, and then analyzed at each time point for the capacity to transport choline (Fig. 1B). As early as 15 min after treatment, the majority of cells began to adhere, and choline transport decreased 22% of its original value, i.e., from 96 ± 13 to 75 ± 6 fmol·mg$^{-1}$·min$^{-1}$ ($P < 0.05$). Further PMA treatments at 30 min, 24 and 48 h resulted in further decreases in choline uptake, corresponding to 47, 66, and 75% of original uptake, respectively (51 ± 3, 32 ± 5, and 25 ± 4 fmol·mg$^{-1}$·min$^{-1}$ at $P < 0.001$, respectively) (Fig. 1B). Taken together, the results in Fig. 1 demonstrate that kinetically, choline transport is represented by two rates in monocytes and PMA-differentiated macrophages. The monocyte transport begins to fall minutes after treatments with PMA, when the cells start to adhere and change to macrophages (46). In fully differentiated cells (24–48 h PMA) the observed choline transport becomes significantly slower, resembling the rates typically observed for simple diffusion.

**Effects of selective transport inhibitors.** Choline transport with the kinetic parameters similar to those characterized in Fig. 1 has been detected in various cells and tissue types outside of neurons (52) and was suggested as a general mechanism to supply choline for phospholipid biosynthesis (52). Before the discovery of CTL1 (11), no choline-specific transporters outside of neurons had been identified having the above characteristics. The nonspecific organic transporters of the OCT family are well characterized, but they transport choline together with other organic cations (49). To investigate whether the measured choline transport in Fig. 1 is specific (CTL1 related) or nonspecific (OCT related), we utilized competition assays with various inhibitors in both untreated monocytes and PMA-derived macrophages. The inhibition results for monocytes are shown in Fig. 2, A and B. The exposure to HC-3, a well-known competitive inhibitor of choline uptake, at 1 µM to target the high-affinity, neuronal-type of transport resulted only in a slight inhibition of choline transport (22%; $P < 0.01$). However, treatments with 200 µM HC-3 to target other HC-3 sensitive transports, such as CTL1-mediated transport, caused an almost complete blockade (94%; $P < 0.001$) relative to the HC-3 untreated cells. The inhibitors of the OCT family of transporters (OCT-1 and OCT-2), such as MPP$^+$ (40 µM) and quinine (50 µM), as well as OCT-2-specific inhibition by the highly potent inhibitor corticosterone (400 nM) (32, 60), showed no significant effects on choline uptake. OCT-3-specific inhibitors were not considered because it has not been demonstrated in the literature that OCT-3 participates in choline transport.

Because choline uptake was substantially lower in 24-h PMA-treated cells (Fig. 1B), we also wanted to assess what type of residual transport is present in macrophages. The results are shown in Fig. 2C. When PMA-derived macrophages were treated with 25 µM HC-3 (∼30% of the $K_m$ value of the transport in macrophages; Fig. 1A), choline uptake significantly decreased (65%; $P < 0.05$). At the same time, the macrophage transport was unaffected by the OCT inhibitor MPP$^+$, showing that this type of transport is not important neither in differentiated nor undifferentiated cells (Fig. 2C). Taken together, the above results imply a choline-specific and HC-3-sensitive transport system distinct from the neuronal transport driven by CHT1, implicating CTL-1 as the most feasible choline transporter in THP-1 cells.

**Identification of transporters expressed in THP-1 cells.** To investigate further what transporters are actually expressed in THP-1 cells and to establish whether they Content change after PMA treatments, mRNA abundance of several putative choline transporters was analyzed after similar PMA treatments as in the kinetic measurements described in Figs. 1 and 2. As shown in Fig. 3, only the HC-3-sensitive and choline-specific CTL1 and the nonspecific OCT-1 mRNA were expressed at time 0, 24, and 48 h PMA treatments. OCT-1 mRNA showed no change in expression with PMA treatments (Fig. 3A). The

![Fig. 2](http://ajpcell.physiology.org/)

Inhibition of choline uptake in THP-1 monocytic and macrophage cells. A: monocyte cells (time 0) incubated simultaneously with both 5 nM [3H]choline and a respective transport inhibitor, 1 µM hemicholinium (HC-3), 200 µM HC-3, and 40 µM 1-methyl-4-phenylpyridinium iodide (MPP$^+$). B: monocyte cells (time 0) incubated simultaneously with both 5 nM [3H]choline and a respective transport inhibitor, 50 µM quinine and 400 nM corticosterone. C: 48-h PMA-treated macrophages incubated similar to monocytes, but with 25 µM HC-3 and 40 µM MPP$^+$. Data in A–C are mean percentage ± SE, where *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ indicate significant differences relative to control. Each time point is representative of four separate experiments and represents fmol of choline uptake per mg of protein per minute.
CTL1 mRNA expression also demonstrates constant levels at all stages of differentiation (Fig. 3B). CHT1 mRNA was not present in THP-1 cells, which was confirmed by using total human brain mRNA control, known to express CHT-1. OCT-2 mRNA was similarly absent, confirmed by using human kidney mRNA control, known to express OCT-2 (Fig. 3A). Together, these results demonstrate that the PMA treatments do not influence transcription of the only present candidate gene transporters, CTL1 and OCT-1. Together with the lack of inhibition with MPP⁺/H11001 and quinine in Fig. 2, A–C, the data show that although expressed, OCT-1 is not quantitatively contributing to the choline transport. Thus the mRNA data generates more support for CTL1 as the sole remaining candidate having suitable kinetic attributes (Figs. 1 and 2) to transport choline in THP-1 cells. To explain the low choline uptake in macrophages (Fig. 1A) because CTL1 mRNA did not change after PMA treatments (Fig. 3B), we hypothesized that the effects are posttranslational, which might include a diminished CTL1 presence at the cell surface and/or the surface CTL1 protein modifications to less active forms.

CTL1 protein expression after PMA treatments. We first utilized Western blot analysis of the total cell lysates to determine whether the total CTL1 protein changed after incubations with PMA. Under such denaturing assay conditions, the human monoclonal CTL1 antibody VIM15 typically detected two or three bands with apparent molecular masses of ~40, 45, and 55 kDa. As shown in Fig. 4A, both monocytes and PMA-derived macrophages display the three bands that only modestly varied in their intensities. These results suggested that the total CTL1 protein is probably not affected by

Fig. 3. mRNA expression of human choline transporter genes in PMA-treated THP-1 cells. First-strand cDNA was synthesized from total RNA extracted differentiating THP-1 monocytes and subjected to RT-PCR analysis. Shown are results obtained from time 0, 24, and 48 h PMA-treated cells. A: mRNA expression of alternate transporters, choline transporter (CHT1), organic-cation transporter (OCT-1), and OCT-2; CHT1 expression was confirmed using human brain cDNA; OCT-1 and OCT-2 expression were confirmed using human kidney cDNA. Time points in THP-1 cells were standardized to β-actin. B: choline transporter-like protein-1 (CTL1) transporter (total) and β-actin control. Time points in THP-1 cells were standardized to β-actin and to time 0 monocyte controls. Blots in A and B represent 3 separate experiments.
the PMA treatments, similarly to the mRNA results (Fig. 3); however, they still do not explain the observed striking reductions in the macrophage choline uptake in Fig. 1.

Because total CTL1 protein did not change, one possibility was to examine whether the CTL1 protein was modified at the cell surface, where it is expected to be actually functioning in choline transport. We performed similar analysis of the plasma membrane CTL1 in both monocytes and PMA-derived macrophages. As shown in Fig. 4B, a single 40-kDa CTL1 band was dominant in monocyte membrane that was completely undetectable in the membranes of the PMA-treated cells. Also, very little or none of the other CTL1 bands (45 and 55 kDa) were detected in the plasma membranes of the PMA-treated cells. The observed changes cannot be attributed to the membrane contamination with other cell compartments because the internal CTL1 content is copiously high in both cell types (Fig. 4A). If a membrane contamination were present, the three CTL1-related bands would appear in the membrane blots, which clearly was not the case (Fig. 4B). Altogether, the Western blot data implied that total CTL1 protein content was not affected by the PMA treatments, but the majority of the membrane CTL1 was reduced after treatments with PMA. The reduced CTL1 membrane content was consistent with the observed reductions in choline transport (Figs. 1 and 2). Those results were undoubtedly confirmed by the FACS analysis of the intact cells, as described in Fig. 5.

Total intracellular CTL1 content was additionally investigated by immunoprecipitation using the same VIM15 antibody. This was different from Western blot analysis, where the total cell proteins were first denatured and then separated before the CTL1-related bands were detected; during immunoprecipitation, the VIM15 antibody first interacted and separated the CTL1 protein from non-denatured cell lysates after which the precipitated CTL1 was detected. As shown in Fig. 4C, the antibody precipitates the intracellular CTL1 as a single protein that also does not change after PMA treatments, confirming the Western blot data as well as the mRNA data (Figs. 3B and 4B). Detection of multiple bands by Western blot analysis (40, 45, and 55 kDa in Fig. 4A) is reproducible and further suggested that such “band shifts” could be caused by differences in posttranslational modifications, most likely by phosphorylation. When the immunoprecipitated CTL1 was probed with phosphoserine antibody (Fig. 4C), the phosphorylation was readily detected in the total cell lysates from monocytes and incrementally increased 1.6- and 1.8-fold after the PMA treatments. In addition, to further investigate this phosphorylation of CTL1, we used the protein phosphatase inhibitor okadaic acid (10). We show in Fig. 4D that PMA-derived macrophages experience a further decrease in choline uptake when treated with okadaic acid compared with untreated control monocytes. Therefore, the inhibition of dephosphorylation results in a state of decreased membrane CTL1 and consequently a decreased choline uptake. Therefore, because the greatest changes were observed in the membrane CTL1 protein content, which decreased below detectable levels after treatments with PMA (Fig. 4B), the membrane CTL1 content was next investigated more precisely on the intact cells in addition to the isolated membranes.

Disappearance of CTL1 from the cell surface. We evaluated the cell-surface CTL1 using FACS analysis. The CTL1 monoclonal antibody VIM15 was extensively tested for similar type of analysis (55). According to the FACS analysis shown in Fig. 5, the CTL1-related fluorescence was at its highest point in undifferentiated monocytes, showing that most cells contained the CTL1 protein on the cell surface (100% specific fluorescence; Fig. 5A). After PMA treatments, CTL1 surface expression decreased and the CTL1-specific peak at 24 h, PMA treatment decreased to only 35% fluorescence relative to the...
control monocytes (Fig. 5C). At 48-h PMA treatment, the CTL1-specific fluorescence was virtually undetectable, with the remaining 10% of the fluorescence relative to monocytes (Fig. 5D). Considering that the PMA treatments did not cause significant changes in total CTL1 mRNA and protein content (Figs. 3B and 4, A and C), the observed decline in the surface fluorescence (Fig. 5E) could only be rationalized by a regulated movement of surface CTL1 to intracellular compartments. This mechanism proposes, as for many other transporters (28, 31), a decrease in the number of functional binding sites and thus choline uptake attenuation in macrophages, in complete agreement with our kinetic analysis (Fig. 1A). Taken together, the disappearance of the CTL1 protein from the cell surface mirrored the reduction of choline uptake, in agreement with the premise that CTL1 is a plasma membrane protein, which regulates choline transport after PMA treatments by protein trafficking, a mechanism well known to similarly govern other transmembrane proteins, including the receptors, ion channels, and transporters (31).

DISCUSSION

The aim of the present study was to investigate the function of human CTL1 in choline transport. The function of CTL1 as a choline transporter has been demonstrated in the Torpedo electric lobe (40), mouse (57), and rat (16, 40, 47). Human CTL1 was cloned and characterized as a cell-surface antigen in monocytes and differentiating dendritic cells; however, its choline transport was not clearly established (55). This study is the first to establish a functional link between the regulation of surface expression of CTL1 protein and choline transport in monocytes and differentiated macrophages. We realized that the well-known induction process of monocytes/macrophages from the PMA/PKC system has the following properties: 1) they contribute to the differentiation processes, 2) they are common modulators of choline uptake in multiple systems, and 3) they are well-documented regulators of surface receptors and transporters (3, 10, 24, 36).

By using kinetic approaches, we demonstrate that choline uptake in monocytes and PMA-differentiated macrophages can be separated into two components that differ in their transport properties. Choline transport for both cell types is protein mediated but is much faster in monocytes and could be inhibited by HC-3, altogether resembling the transport established in cells transiently expressing the mouse (57) and rat (16, 47) CTL1 homologues and unrelated to the transport regulated by CHT1. We further established that THP-1 monocytes and macrophages express mRNAs for only two candidate transporters, CTL1 and the organic-cation transporter OCT-1, Competition studies (14, 32, 61) with MPP+, quinone, or corticosterone, which selectively block the OCT transporters, produced no effect on choline transport, and the OCT-1 was consequently eliminated from further consideration. Thus, based on the kinetic, inhibitory, and mRNA data, we focused on CTL1 as the best candidate for regulating choline transport in monocytes and PMA-derived macrophages. We found no significant changes in total CTL1 protein and mRNA after PMA treatments suggesting that significant reduction in choline transport in differentiated macrophages could be a result of reduced amount of surface CTL1 protein. By using flow cytometry analyses on intact cells, we further demonstrated that the plasma membrane CTL1 was dramatically reduced after PMA treatments. In fact, considering technical discrepancies between the FACS analysis and cell radiolabeling methodologies, the rate of disappearance of the CTL1 protein from the cell surface, and the reduction of choline uptake had similar trends during the 24-h PMA treatments, both had become significantly diminished in macrophages, in agreement with an increased CTL1 internalization from the cell surface, generally a common mechanism for the regulation of number of cell membrane proteins (31).

Neuronal choline transport with CHT1 has been shown to be downregulated by PMA with subsequent decrease in choline uptake, as demonstrated in both Limulus brain hemi-slices, and hippocampal and striatal synaptosomes (8, 56). These results were both confirmed to be acute PKC-related events. Our results provide evidence for an inhibitory effect of PMA on CTL1, substantiated by the lack of choline uptake after short-term PMA treatments, shown in Fig. 1A. Interestingly, CTL1 surface expression at 30-min PMA treatment, as shown in Fig. 5, was not significantly decreased. Therefore, these results imply the possibility of PKC regulation of CTL1, which has immediate effect on choline uptake, as is the case with CHT1 (10). Our immunoprecipitation experiments demonstrate an increased phosphorylation of CTL1 in total cell lysates after 24- and 48-h PMA treatments. The phosphorylated CTL1 was not detected at the cell surface and considering the choline transport measurements, our data suggest that the phosphorylated forms most likely represent an intracellular, inactive pool of CTL1; however, this needs to be further substantiated in future experiments. Very important to note is emerging research altering the long-standing view that phorbol esters affect only PKC cascade because in addition they could also regulate protein kinase D, GTPase-activating proteins, diacylglycerol kinases, and still others (21, 48). Although most reviews suggest that long-term PMA treatment will lead to an increased proteolysis of PKC isoforms, potentially leading toward more differentiated cells, earlier results did not demonstrate this phenomenon (46). Instead PKC isoform expressions (α, β, δ, and ε) in THP-1 cells persisted after 72 h of PMA treatment, suggesting that PKC mechanisms still could be valid in the regulation of phosphorylation and trafficking of CTL1, as described in this study.

We examined the CTL1 protein sequence (GeneBank accession no. AJ727365) for putative PKC phosphorylation sites. On the basis of the most probable model for the membrane topology of this transporter, there are four cytosolic serine residues to be considered. This model also suggests that CTL1 has 10 transmembrane domains with intracellular COOH and NH2 termini, which coincides with the previous models (40, 55). Although models could vary in their transmembrane domain predictions with some overlaps, the internal PKC sites that remain consistent are the sites at the NH2 (amino acids 13-15) and COOH terminus (amino acids 629-631). In addition, other studies (23, 35) have found that PKC commonly phosphorylates transmembrane proteins at the COOH and NH2 terminus, suggesting a high possibility that these terminal sites are regulatory.

Similar regulation mechanisms have been frequently reported for numerous neurotransmitter transporters such as GABA, serotonin, and glutamate (5, 20, 22). Many of these
transporters had shown decreased transport activity and increased intracellular trafficking upon PMA treatments (45), in accordance with the data presented in this study. Although few long-term PMA studies (7, 25) have been performed on choline or neurotransmitter transporters, similar findings were seen in the effects of PMA on the β2- and β3-adrenergic receptors. In a recent experiment, a time-dependent decrease in protein expression and ligand binding of the β2-adrenergic receptor was seen in PMA treated C6 glioma cells, with a maximal decrease at 24 h (~50% of control) (25). Site-directed mutagenesis on the α2A-adrenergic receptor found a PKC serine-phosphorylation site is linked with desensitization of the receptor to its ligand and its internalization (26). Many of the results involving the major neurotransmitter transporters have established, however, that although PKC does work to modulate transporter cell surface expression, its primary modulation is not in the direct phosphorylation of the enzyme (6, 20). As well, our experiments worked to draw such a conclusion for CTL1 that involves a common mechanism associated with changes in function that involves protein redistribution at the plasma membrane and possibly the presence of an inactive intracellular pool of the CTL1 protein as a mechanism of regulating its transport activity.

Previous reports (55) have further shown that CTL1 is a surface antigen that may also serve an immunological function and noted a downregulation on the cell surface of differentiating dendritic cells with the addition of ionomycin, forming highly immune-responsive cells. The decrease in CTL1 surface expression was consequently reversed by the immunosuppressive cytokine IL-10 (55). Similarly, IL-10 secretion could be induced by CTL1 antibody in LPS-activated dendritic cells. In conjunction with these results, our finding of diminished CTL1 presentation at the cell surface of differentiating macrophages is very interesting, especially considering inflammatory aspects of atherosclerosis, triggered by proinflammatory cytokines and monocyte-endothelium cell adhesions. A stimulation of IL-10 secretion from macrophages has proven to inhibit the advent of these interactions and could be ultimately involved in prevention of the formation of foam cells and atherosclerosis (43, 53). Future research should continue to investigate the potential relationships between choline transport, CTL1 presentation, and IL-10 production in monocyte/macrophage cells because it may pertain to the anti-inflammatory role of CTL1. It has also been shown that CTL1 is upregulated by retinoic acid in myeloid to neutrophil cells and downregulated on myeloid to monocyte-endothelium cell adhesions. A stimulation of IL-10 secretion from macrophages has proven to inhibit the advent of these interactions and could be ultimately involved in prevention of the formation of foam cells and atherosclerosis (43, 53).

All together, this study demonstrates more strongly the functional role of CTL1 as a choline transporter. It is also the first to shed new light on the regulation of this transporter by protein trafficking from the cell surface. Future investigations should continue to pursue the importance of this transporter in immune cell development and response because it may give way to critical leads into the prevention of chronic inflammatory diseases such as atherosclerosis.

ACKNOWLEDGMENTS

We gratefully acknowledge the contributions made by Daniel Cole and Angela Tie, and thank Lyn Hillyer for assistance with flow cytometry analyses.

GRANTS

This study was supported by an Operating Grant from the Natural Sciences and Engineering Research Council of Canada and the Ontario Government Premier Research Excellence Award (both to M. Bakovic).

REFERENCES


