Induction of the differentiation of HL-60 cells by phorbol 12-myristate 13-acetate activates a Na⁺-dependent uridine-transport system

Involvement of protein kinase C

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The Na⁺-dependent transport and facilitated diffusion of uridine were measured after differentiation of HL-60 leukaemia cells along the monocytic pathway by phorbol 12-myristate 13-acetate (PMA). PMA (200 ng/ml) caused a marked increase in Na⁺-dependent uridine transport within 48 h of exposure that was attributable to an increase in transport affinity (apparent K_m values of 1.15 ± 0.22 and $44 \pm 4.4 \mu\text{M}$ for PMA-induced and uninduced cells respectively), with no change in V_{max} (0.15 ± 0.02 and 0.13 ± 0.01 pmol/s per μl of cell water for PMA-induced and uninduced cells respectively). A corresponding rapid decrease in both the rate of facilitated diffusion and the formation of uracil nucleotides occurred in PMA-induced cells. As a consequence of these changes, intracellular pools of uridine 3–4-fold greater than those in the medium were generated. A similar increase in Na⁺-dependent transport of adenosine, inosine, guanosine, thymidine and cytidine (K_m values of 1–4 μM) was observed. The effects of PMA on the activation of the Na⁺-dependent uridine transporter were inhibited by staurosporine, suggesting the involvement of protein kinase C. The findings indicate that a change in the balance of the cellular mechanisms employed for nucleoside transport occurs during the monocytic differentiation of HL-60 leukaemia cells.

INTRODUCTION

The HL-60 human promyelocytic leukaemia, a well-characterized cell line that can be induced to differentiate terminally into granulocyte-like (Collins *et al.*, 1977; Gallagher *et al.*, 1979) or monocyte-like (Lotem & Sachs, 1979; Rovera *et al.*, 1979; Miyaura *et al.*, 1981) cells by a variety of agents, possesses a very low level of Na⁺-dependent active transport and considerable NBMPR-sensitive facilitated diffusion for uridine (Lee *et al.*, 1990b). Since only low levels of Na⁺-dependent uridine transport exist in undifferentiated HL-60 cells, they are unable to generate intracellular uridine concentration gradients. This phenomenon is attributable to the high activity of the facilitated-diffusion system, which equilibrates the concentration of uridine across membranes, as well as to the rapid metabolism of uridine.

When HL-60 cells were induced to differentiate along the granulocytic pathway by dimethyl sulphoxide, a marked increase in Na⁺-dependent uridine transport occurs, which is attributable to a 28-fold increase in transport affinity, with no change in V_{max} (Lee *et al.*, 1990b). Simultaneously, a 10–20-fold decrease in the rate of facilitated diffusion for nucleosides is observed in mature cells. This decrease in the rate of facilitated diffusion is attributable to a decrease in the number of transport sites per cell, as indicated by a parallel decrease in the number of high-affinity NBMPR-binding sites on the cell surface (Chen *et al.*, 1986; Lee *et al.*, 1990b). The balance of these changes generated a 10–15-fold increase in the intracellular concentration of uridine relative to that in the medium at a physiological concentration of 1 μM -uridine. These results are in contrast with the findings with undifferentiated HL-60 cells or to those with a large number of

other neoplastic cell lines (Darnowski & Handschumacher, 1986). Previously, accumulations of free uridine had only been reported in normal tissues such as kidney, liver, spleen and intestine (Darnowski & Handschumacher, 1986), a result attributable to the Na⁺-dependent nucleoside-transport system(s) (Schwenk *et al.*, 1984; Darnowski *et al.*, 1987; Jarvis, 1989; Jarvis *et al.*, 1989; Lee *et al.*, 1989, 1990a). The findings suggest that the loss or marked decrease of Na⁺-dependent transporter activity may coincide with the development of the malignant state.

The lack of discrete localization of the facilitated diffusion and Na⁺-dependent transport carriers in different portions of the cell membrane of the differentiated HL-60 cell line and normal splenocytes must be contrasted with the compartmentalization of the two transport mechanisms in renal (Lee *et al.*, 1989, 1990a) and intestinal (Jarvis, 1989) epithelial cells. This finding suggests that the Na⁺-dependent uridine transport is essential to a physiological function within these cells rather than a means for absorption in a unidirectional manner. The physiological role(s) of intracellular uridine pools in normal and terminally differentiated leukaemia cells, however, is not known. Uridine may play a regulatory role in cell transformation in addition to its role as a precursor in the salvage pathway for pyrimidine nucleotide synthesis. In the present report, we have expanded our previous study (Lee *et al.*, 1990b) by reporting the increased activity of Na⁺-dependent uridine transport in HL-60 cells induced to differentiate along the monocytic pathway by PMA. We also describe the effects of the modulation of the differentiation process by staurosporine. The results demonstrate that the increase in Na⁺-dependent uridine transport and the subsequent expansion of intracellular uridine pools, previously shown with

Abbreviations used: NBMPR, nitrobenzylthioinosine; PMA, phorbol 12-myristate 13-acetate.

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granulocytic differentiation of HL-60 leukaemia cells, also occur when phenotypic differentiation to monocytes/macrophages is induced.

EXPERIMENTAL

Cell culture

HL-60 promyelocytic leukaemia cells were kindly provided by Dr. Robert C. Gallo of the National Cancer Institute, Bethesda, MD, U.S.A. The HL-60 cultures used in these experiments were expanded from stocks frozen in liquid N₂, and consisted of cells between passages 25 and 65. Cell stocks were routinely checked for mycoplasma contamination by the gene-probe method (Gen-Probe Inc., San Diego, CA, U.S.A.). Cells were grown in continuous suspension in RPMI 1640 medium (GIBCO, Grand Island, NY, U.S.A.) supplemented with 15% (v/v) heat-inactivated (50 °C for 30 min) fetal-calf serum (GIBCO), 50 units of penicillin/ml and 50 µg of streptomycin/ml (Sigma Chemical Co., St. Louis, MO, U.S.A.). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in air. Cell differentiation was induced by suspending 50 ml of HL-60 cells at an initial density of 2×10^5 cells/ml in 175 cm² Corning plastic flasks in the presence of various concentrations of PMA for the indicated time intervals. Cell numbers were determined with a Coulter particle counter equipped with a Channelizer (Coulter Electronics, Hialeah, FL, U.S.A.).

Assessment of differentiation

The capacity of HL-60 cells to undergo functional maturation after exposure to PMA in the absence or presence of 40 nm-staurosporine was determined by monitoring cell morphology and adhesion to flasks (Rovera *et al.*, 1979).

Transport studies

To prepare cells for transport studies, a suspension of cells was centrifuged at 1000 *g* for 5 min in a Sorvall GLC-4 centrifuge. The pellet was suspended in 50 ml of Na⁺-free (choline replacement) Hanks' balanced salts medium plus 5.5 mM-D-glucose and 4 mM-Hepes buffer (pH 7.4), and centrifuged at 1000 *g* for 5 min. The pellet was resuspended with an appropriate volume of transport medium to give a final cell density of $(6-7) \times 10^7$ cells/ml. This procedure decreased the extracellular Na⁺ content from 140 mM to less than 0.1 mM as measured by flame photometry.

The uptake of [³H]uridine, [³H]adenosine, [³H]inosine, [³H]guanosine, [³H]cytidine and [³H]thymidine was initiated by mixing 30 µl of cell suspension containing 2×10^6 cells (with or without 10 µM-NBMPR) with 60 µl of radioactive substrate (with or without Na⁺) in a 1.5 ml Eppendorf microfuge tube at 22–24 °C. The detailed composition of the final incubation medium is given in each Figure legend. Na⁺-dependent uridine transport is operationally defined as the difference between the intracellular content of [³H]uridine in cells treated with 10 µM-NBMPR to block facilitated diffusion in the presence and absence (choline replacement) of Na⁺. Facilitated diffusion of uridine is defined as the difference between the intracellular content of [³H]uridine in cells suspended in Na⁺-free medium (choline replacement) in the presence and absence of NBMPR. A 60 µl portion of the mixture was placed in an 'oil-stop tube', consisting of a 400 µl Eppendorf microfuge tube containing 125 µl of oil (16% Fisher 0121 light paraffin oil and 84% Dow-Corning 550 silicone fluid, with a final density of 1.04 g/ml) layered over 30 µl of 15% (v/v) trichloroacetic acid. After an appropriate time interval, cells were removed from the transport medium by centrifugation into the trichloroacetic acid, by using a Beckman model B microfuge for 30 s at 10000 *g*. Microfuge tubes were

then cut through the oil layer, and both the supernatant and the pellet were placed in plastic mini scintillation vials. To each sample 5 ml of Ecolite (ICN, Cleveland, OH, U.S.A.) was added, and the mixtures were vortex-mixed vigorously. Radioactivity was determined in a Beckman model LS 7000 liquid-scintillation spectrometer. Approximate initial rates of Na⁺-dependent uridine transport were determined by using a 6 s time interval. 'Time-zero' values for uptake, attributed to radioactivity trapped in the cell pellet, were determined by centrifuging 20 µl of cell suspension through a layer of radioactive substrate (40 µl) layered over the oil in an oil-stop tube. These values were subtracted from measurements of uptake by HL-60 cells. The intracellular volume of HL-60 cells was calculated in all experiments by using ³H₂O to determine total water space, and [¹⁴C]inulin was employed to calculate extracellular space (Darnowski *et al.*, 1987). Kinetic constants for Na⁺-dependent uridine transport were determined by non-linear least-squares fits of the Michaelis-Menten equation by using the computer program ENZFITTER (Leatherbarrow, 1987).

Intracellular free uridine

Cells were incubated with 5 µM-[³H]uridine at 22 °C for 15 s and 300 s time intervals. Reactions were terminated by the oil-stop method described above. The cell pellets from identical time points were pooled and extracted with an equal volume of 1 M-triethylamine in Freon (Darnowski & Handschumacher, 1986). The form of ³H radioactivity in the cell pellet was assessed by h.p.l.c. using a C₁₈ reverse-phase column. The mobile phase consisted of 10 mM-H₃PO₄ and 30 µM-heptanesulphonic acid (pH 3.3), with a flow rate of 1 ml/min at 13 °C. Under these conditions, the following retention times were observed: void volume 3 min, uracil nucleotides 4–5 min, uracil 10 min and uridine 16 min.

Chemicals

[5-³H]Uridine (20 Ci/mmol), [2-³H]inosine (35 Ci/mmol), [2,8-³H]adenosine (44 Ci/mmol), [methyl-³H]thymidine (63 Ci/mmol), [8-³H]guanosine (5 Ci/mmol) and [5-³H]cytidine (26 Ci/mmol) were purchased from Moravak Biochemicals Inc. (Brea, CA, U.S.A.). ³H₂O (18 µCi/mmol) was obtained from New England Nuclear Corp. (Boston, MA, U.S.A.) and [¹⁴C]inulin (10 mCi/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.). Staurosporine and PMA were obtained from Sigma Chemical Co. All other reagents were of analytical grade.

RESULTS

Effects of various concentrations of PMA on the transport of uridine by HL-60 cells

HL-60 cells in early exponential growth were exposed to various concentrations of PMA (50–100 ng/ml) for 48 h. Changes in the ability to transport a concentration of uridine that approached physiological conditions (5 µM) via the facilitated-diffusion mechanism (choline-containing medium) and Na⁺-dependent active transport (Na⁺- and NBMPR-containing medium) were measured.

The facilitated diffusion of 5 µM-uridine in HL-60 cells, measured at a 15 s uptake interval, decreased rapidly after exposure to 50 ng of PMA/ml and reached a minimum at 200 ng of PMA/ml (Fig. 1a). In contrast, a corresponding increase in the rate of Na⁺-dependent uridine transport was observed. The maximum increase in Na⁺-dependent uridine transport activity was achieved at 100 ng of PMA/ml. At this concentration, the rate of Na⁺-dependent uridine uptake in HL-60 cells exposed to PMA was about 4-fold higher than that of uninduced leukaemia

Fig. 1. Effects of PMA concentration on uridine transport and utilization in HL-60 cells

HL-60 cells at an initial concentration of 2×10^5 cells/ml in RPMI 1640 medium were incubated with various concentrations of PMA (0–1000 ng/ml) for 48 h. Cells were harvested, washed and in panel (a) assessed for [³H]uridine (5 μM) uptake by facilitated diffusion (□, uptake in the absence of Na⁺) and Na⁺-dependent transport (■, uptake in the presence of Na⁺ and 10 μM-NBMPR) at a 15 s uptake interval. In panel (b) intracellular [³H]uridine pools were measured after exposure to 5 μM-[³H]uridine for 5 min. Intracellular free uridine concentrations were obtained by h.p.l.c. analysis of intracellular radioactivity in this nucleoside. The data were corrected for radioactivity associated with cell membranes at zero time of uptake. Abbreviations: Na + N, Na⁺ plus NBMPR; C, choline.

Fig. 2. Effects of time of PMA exposure on the transport and intracellular accumulation of uridine in HL-60 cells

HL-60 cells at the initial concentration of 2×10^5 cells/ml in RPMI 1640 medium were incubated with 200 ng of PMA/ml from 0 to 72 h. At the indicated time intervals, cells were harvested, washed and assessed in panel (a) for [³H]uridine (5 μM) uptake by facilitated diffusion (□, uptake in the absence of Na⁺) and Na⁺-dependent transport (■, uptake in the presence of Na⁺ and 10 μM-NBMPR) at a 15 s uptake interval. In panel (b) intracellular [³H]uridine pools were measured after exposure to 5 μM-[³H]uridine for 5 min. Intracellular free uridine concentrations were obtained by h.p.l.c. analysis of intracellular radioactivity in this nucleoside. The data were corrected for zero-time uptake. Abbreviations: Na + N, Na⁺ plus NBMPR; C, choline.

cells. The significance of this change in the rate of Na⁺-dependent uridine transport can be seen in the formation of intracellular uridine pools measured 5 min after incubation of HL-60 cells with [³H]uridine. A 3-fold concentration gradient of free uridine in cells versus the medium was observed (Fig. 2b). This concentration gradient of uridine was not observed in uninduced cells, and was not generated by the facilitated-diffusion mechanism.

Effects of the time of exposure to PMA on the transport of uridine by HL-60 cells

Exponentially growing HL-60 leukaemia cells were exposed to 200 ng of PMA/ml for 0–72 h to assess the temporal relationship of these changes. Fig. 2(a) shows that the facilitated diffusion of uridine decreased progressively with time in HL-60 cells after exposure to 200 ng of PMA/ml. The amount of intracellular uridine associated with cells treated with PMA for 72 h, when only facilitated diffusion could occur, was about 20% of the amount found with uninduced HL-60 cells when a short uptake interval of 15 s was employed. In contrast, the approximate initial rate of Na⁺-dependent uridine transport increased substantially after an 8–24 h exposure of HL-60 cells to PMA, and reached a maximum at 48 h. After 48 h of continuous treatment with PMA, the rate of Na⁺-dependent uridine flux was about 3-fold higher than that for uninduced cells. As a result, an intracellular accumulation of uridine occurred that was 3–4-fold greater than that present in the medium (Fig. 2b). This concentration gradient of uridine was not observed within the first 24 h of exposure to PMA, and was completely abolished by the absence of Na⁺ (choline replacement).

Time course of [³H]uridine uptake by PMA-induced HL-60 cells

Fig. 3 demonstrates that total uptake of 5 μM-[³H]uridine in PMA-induced HL-60 cells in the presence of Na⁺ was at least 2-fold greater than the uptake in the absence of Na⁺ (choline replacement) at a 5 min uptake interval. Since uptake of uridine in the absence of Na⁺ was completely inhibited by NBMPR, the facilitated-diffusion process appears to be the only operative system under these conditions. In contrast, even after 20 min, the total amount of intracellular radioactivity in cells exposed to Na⁺ was slightly higher in the presence of NBMPR (65.5 pmol/μl) than in the absence of NBMPR (61.8 pmol/μl), despite the initial decrease in uridine influx. The uptake of [³H]uridine in the

Fig. 3. Time course of uridine uptake by PMA-induced HL-60 cells

HL-60 cells treated with 200 ng of PMA/ml for 48 h were incubated with (○, ●) or without (□, ■) 10 μM-NBMPR for 5 min before exposure to [³H]uridine (final concn. 5 μM) in the presence (■, ●) or absence (□, ○) of Na⁺ (choline replacement). At appropriate time intervals, uptake was terminated by the oil-stop method described in the Experimental section. Data are from a representative experiment of three separate experiments. Values are means $\pm$ S.E.M. of triplicate estimates; where absent, the S.E.M. was smaller than the symbol. Abbreviations: Na, Na⁺; C, choline; N, NBMPR.

presence of Na⁺ and NBMPR was linear for at least 15 s at 22 °C. Therefore, in subsequent kinetic experiments, approximate initial rates of Na⁺-dependent uridine uptake were determined by measuring the cellular content of uridine after incubation intervals of 6 s at 22 °C.

Kinetics of Na⁺-dependent uridine transport in PMA-induced HL-60 cells

Na⁺-dependent uridine-influx rates exhibited by HL-60 cells exposed to 200 ng of PMA/ml for 48 h were resolved into two components: (a) a linear component and (b) a saturable component which obeyed Michaelis–Menten kinetics. The saturable transport component, estimated in the presence of Na⁺ and NBMPR, after subtraction of the linear component, estimated in the absence of Na⁺ by using choline replacement and in the presence of NBMPR, is shown in Fig. 4. These kinetic studies suggest that the increase in Na⁺-dependent uridine-transport activity in PMA-induced cells is consequent to a 38-fold enhancement in transport affinity ($K_m = 1.15 \pm 0.22 \mu\text{M}$, $V_{\text{max}} = 0.15 \pm 0.02 \text{ pmol/s per } \mu\text{l}$ of cell water; $n = 3$), with no significant change in V_{max} , as compared with uninduced cells ($K_m = 44 \pm 4.4 \mu\text{M}$, $V_{\text{max}} = 0.13 \pm 0.01 \text{ pmol/s per } \mu\text{l}$ of cell water; Lee *et al.*, 1990b).

In addition to measurements of the initial rates of uridine transport, it was considered relevant to assess the physiological consequence at later times. After a 5 min incubation with 5 μM -[³H]uridine, the entry of radioactivity into the intracellular [³H]uracil nucleotide pools in Na⁺-free medium was 8 times greater in uninduced (101 pmol/ μl) than in PMA-induced HL-60 cells (12 pmol/ μl) (Table 1). Nevertheless, the concentration of intracellular free uridine remained at 5 pmol/ μl under these conditions where transport is totally dependent upon facilitated diffusion. In contrast, the levels of intracellular [³H]uracil nucleotides present in both uninduced and PMA-induced cells utilizing the Na⁺-dependent transport process (Na⁺ plus NBMPR me-

dium) were low (less than 5 pmol/ μl) and were not significantly different from each other. This low level of intracellular [³H]uracil nucleotides in uninduced cells was due to the slow entry of [³H]uridine by the Na⁺-dependent uridine transporter. Under these conditions, [³H]uracil nucleotides constituted over 95 % of the total intracellular non-uridine radioactive metabolites. Thus, despite increased intracellular pools of uridine, a marked decrease in uridine anabolism was seen in cells that were induced to differentiate along the monocytic pathway.

Table 1. Effects of staurosporine on intracellular uridine and uracil nucleotide pools of PMA-induced HL-60 cells

HL-60 cells treated with or without 200 ng of PMA/ml for 48 h in the absence or presence of 40 nM-staurosporine (STSP) were assayed for intracellular free [³H]uridine concentrations and intracellular [³H]uracil nucleotide concentrations by h.p.l.c. after incubation with 5 μM -[³H]uridine for 5 min. Na⁺-dependent transport was measured in the presence of Na⁺ and 10 μM -NBMPR. Facilitated diffusion was measured in the absence of Na⁺ (choline replacement) and NBMPR. Data are means $\pm$ S.E.M. of three separate experiments, and were corrected for zero-time uptake.

	Na ⁺ -dependent transport	Facilitated diffusion
Uridine (pmol/ μl)		
Control	1.4 $\pm$ 0.1	6.1 $\pm$ 0.8
PMA	15.4 $\pm$ 1.9	4.5 $\pm$ 0.4
PMA + STSP	1.2 $\pm$ 0.1	5.2 $\pm$ 0.6
Uracil nucleotides (pmol/ μl)		
Control	4.1 $\pm$ 0.5	100.9 $\pm$ 13.1
PMA	9.2 $\pm$ 1.2	11.8 $\pm$ 1.4
PMA + STSP	4.6 $\pm$ 0.6	75.2 $\pm$ 10.1

Fig. 4. Concentration-dependence of Na⁺-dependent uridine transport by PMA-induced HL-60 cells, and staurosporine-sensitivity

[³H]Uridine transport was determined in HL-60 cells treated with 200 ng of PMA/ml for 48 h in the absence (●) or presence (□) of 40 nM-staurosporine (STSP). After 6 s, Na⁺-dependent uridine transport (in the presence of Na⁺ and 10 μM -NBMPR) was still linear and was terminated as described in the Experimental section. The data shown were corrected for the linear component of uridine transport determined in the Na⁺-free (choline replacement) NBMPR-containing medium. Values are means $\pm$ S.E.M. of three separate experiments performed in triplicate; where absent, the S.E.M. is smaller than the symbol. The kinetic constants for the data shown are $K_m = 1.15 \pm 0.22 \mu\text{M}$ and $V_{\text{max}} = 0.15 \pm 0.02 \text{ pmol/s per } \mu\text{l}$ of cell water for PMA-induced cells, and $K_m = 38 \pm 1.5 \mu\text{M}$ and $V_{\text{max}} = 0.15 \pm 0.03 \text{ pmol/s per } \mu\text{l}$ of cell water for PMA-induced cells treated with 40 nM-staurosporine. The kinetic curve ($K_m = 44 \pm 4.4 \mu\text{M}$ and $V_{\text{max}} = 0.13 \pm 0.01 \text{ pmol/s per } \mu\text{l}$ of cell water) for uninduced cells (●) (Lee *et al.*, 1990b) was plotted for comparison.

Fig. 5. Effects of PMA and staurosporine on the proliferation and differentiation of HL-60 cells

HL-60 cells treated with PMA from 0 to 72 h in the absence (●) or presence (□) of 40 nM-staurosporine (STSP) were assayed in (a) for growth and in (b) for substrate adherence as described in the Experimental section. Values are means $\pm$ S.E.M. of triplicate determinations from a representative experiment of two separate experiments; where absent, the S.E.M. was smaller than the symbol.

Assessment of monocytic differentiation

The effects of PMA on the differentiation of HL-60 cells were studied by monitoring both the growth and the number of cells that were attached to culture flasks. Under the conditions employed, 200 ng of PMA/ml caused approx. 35 % of the cells to attach to the plastic substrate after 24 h (Fig. 5b), often in small clumps. After 48 h of continuous treatment with PMA, more than 80 % of the cells were adherent, whereas uninduced cells remained as suspensions of single cells in culture. Although the adherent cells were viable, they were not able to proliferate, as indicated by a marked decrease in growth rate (less than 13 % of that of uninduced cells after 72 h; Fig. 5a). Cells that remained in suspension after 72 h of exposure to PMA, however, were not viable.

Effects of staurosporine on induction of the Na⁺-dependent transport of uridine

It is generally acknowledged that protein kinase C is the primary target of the phorbol esters (Nishizuka, 1984). The involvement of protein kinase C in the PMA-induced effects on uridine uptake was evaluated by examining the effects of staurosporine, a potent inhibitor of protein kinase C, on the changes in Na⁺-dependent uridine transport elicited by PMA. The increase in Na⁺-dependent transport affinity for uridine produced by PMA was virtually eliminated by this inhibitor ($K_m = 38 \pm 1.5 \mu\text{M}$, $V_{\text{max}} = 0.15 \pm 0.03 \text{ pmol/s per } \mu\text{l}$, $n = 3$; Fig. 4). As a result, no generation of intracellular uridine pools was observed (Table 1). In contrast, this inhibitor of protein kinase C had no detectable effect on uridine metabolism and transport in uninduced cells (results not shown). Furthermore, in the presence of staurosporine, the ability of PMA-induced cells to anabolize uridine remained essentially the same (75 %) as in wild-type HL-60 cells (Table 1). As expected, 40 nM-staurosporine abolished the changes in cell adherence (i.e. differentiation) that occurred after treatment with PMA, with less than 10 % of the cells being attached after 72 h. Non-attached cells were viable and continued to proliferate. As a result, a higher growth rate (50 % of that of uninduced controls at 72 h) was observed (Fig. 5a).

Effects of PMA on the transport kinetics of other nucleosides

Adenosine, inosine, guanosine, cytidine and thymidine were also effective substrates of the Na⁺-dependent transport system in

Fig. 6. Time course of nucleoside uptake by HL-60 cells after exposure to PMA

HL-60 cells treated with 200 ng of PMA/ml for 48 h were incubated without (□, ■) or with (○, ●) 10 μM -NBMPR for 5 min before addition of [³H]adenosine, [³H]inosine, [³H]guanosine, [³H]cytidine or [³H]thymidine (final concn. of each 5 μM) in the presence (■, ●) or absence (□, ○) of Na⁺ (choline replacement). After an appropriate time interval, uptake was terminated as described in the Experimental section. The data in each panel are from a representative experiment of at least two separate experiments. Values are means $\pm$ S.E.M. of triplicate estimates; where absent, the S.E.M. was smaller than the symbol. Abbreviations: Na, Na⁺; C, choline; N, NBMPR.

Table 2. Kinetic constants of Na⁺-dependent transport of adenosine, inosine, guanosine, cytidine and thymidine by PMA-induced HL-60 cells

Uptake was initiated by the addition of [³H]adenosine, [³H]inosine, [³H]guanosine, [³H]cytidine or [³H]thymidine in the presence of Na⁺ and 10 μM -NBMPR to cells treated with PMA (200 ng/ml) for 48 h. After 6 s at room temperature, uptake was terminated as described in the Experimental section. Data were corrected for Na⁺-independent NBMPR-insensitive flux determined in Na⁺-free medium (choline replacement) and in the presence of 10 μM -NBMPR. Values are means $\pm$ S.E.M. of at least two separate experiments, each performed in triplicate.

Nucleoside	K_m (μM)	V_{max} (pmol/s per μl of cell water)
Adenosine	1.1 ± 0.2	0.12 ± 0.02
Inosine	2.1 ± 0.1	0.17 ± 0.03
Guanosine	2.0 ± 0.5	0.18 ± 0.02
Cytidine	1.8 ± 0.2	0.18 ± 0.01
Thymidine	3.5 ± 0.9	0.18 ± 0.03

PMA-induced HL-60 cells, with apparent K_m values ranging from 1 to 4 μM and V_{max} values of 0.12–0.18 pmol/s per μl of cell water (Table 2). The similarity of these values to the K_m for uridine transport (Fig. 4) suggests that these nucleosides may share the same Na⁺-dependent transport system with uridine in differentiated HL-60 cells.

Although [³H]uridine uptake in PMA-induced HL-60 cells in the presence of Na⁺ was slightly enhanced by NBMPR, no enhancement of the accumulation of adenosine, inosine, guanosine or cytidine was observed after 5 min (Fig. 6) or up to 20 min

(results not shown) in the presence of NBMPR. One possible explanation for these findings was the rapid metabolism of these nucleosides, especially purine nucleosides, into metabolites that readily diffused from cells via an NBMPR-sensitive mechanism(s). Unlike the pyrimidine nucleosides, a considerable cellular accumulation of purine-associated radioactivity was detected when cells were incubated in medium containing NBMPR in the absence of Na⁺ (choline replacement). This phenomenon may reflect re-utilization of free purine bases after the action of purine nucleoside phosphorylase. Such a possibility requires investigation.

DISCUSSION

In the present study, we observed a marked increase in Na⁺-dependent uridine-transport activity in HL-60 leukaemia cells upon monocytic differentiation induced by PMA. This increase in Na⁺-dependent transport activity was attributed to a 38-fold increase in the apparent transport affinity and appeared to be a consequence of the differentiation process rather than to a non-specific effect of the inducer on the plasma membrane or on the transporter protein itself. That the granulocytic inducers of HL-60 maturation, retinoic acid, selenazole and dimethyl sulphoxide, also increased Na⁺-dependent uridine transport in HL-60 cells (Lee *et al.*, 1990b; C. W. Lee, J. A. Sokoloski, A. C. Sartorelli & R. E. Handschumacher, unpublished work) support this conclusion. Furthermore, the increase in Na⁺-dependent transport affinity for uridine produced by PMA was essentially eliminated by staurosporine. Thus changes in Na⁺-dependent transport activity appeared to be specifically linked to the overall process of differentiation and were not dependent upon the particular agent used to induce differentiation.

Another similarity of the present investigation to previous studies using dimethyl sulphoxide-induced HL-60 cells was the inability of NBMPR, a potent inhibitor of facilitated nucleoside transport, to enhance the accumulation of adenosine, guanosine, inosine and cytidine in PMA-induced HL-60 cells. It appears that a single Na⁺-dependent nucleoside-transport system with different substrate specificities exists in differentiated HL-60 cells, since the Na⁺-dependent uridine transporter that exists in cells of either the granulocytic or monocytic phenotype is capable of transporting each of the nucleosides (i.e. adenosine, guanosine, inosine, cytidine and thymidine) with about equal affinity ($K_m = 2\text{--}5\ \mu\text{M}$).

Although the molecular events critical for the PMA-induced differentiation of HL-60 cells are still a matter of conjecture, there is considerable evidence to support the involvement of protein kinase C in this process. To date, protein kinase C is the only documented receptor for PMA (Nishizuka, 1984), and initial effects of PMA on HL-60 cells appear to involve the translocation of cytoplasmic protein kinase C to the plasma membrane, an event that results in the depletion of the cytosolic enzyme and a corresponding increase in the membrane-bound enzyme (Shoji *et al.*, 1986, 1987). Although the functional

consequences of this distributional change in protein kinase C is unclear, it is possible that the translocation results in increased phosphorylation of plasma-membrane proteins (Kiss *et al.*, 1987a,b), which may be essential for initiation of the commitment to enter a differentiation pathway. The identities and functions of phosphoproteins and their contribution to the eventual attainment of the terminally differentiated state of leukaemia cells remain to be identified. In light of the known ability of PMA to stimulate protein kinase C, and the sensitivity of Na⁺-dependent transport of uridine to staurosporine, it is conceivable that the Na⁺-dependent uridine transporter is one of the cellular proteins that are phosphorylated by protein kinase C. Such an event may be essential, or at least intimately related, to the differentiation process.

This research was supported by Grant CH67 from the American Cancer Society and by U.S. Public Health Service Grants CA-02817 and CA-45303 from the National Cancer Institute.

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