Enhancement of pertussis-toxin-sensitive Na^+-dependent uridine transporter activity in HL-60 granulocytes by *N*-formylmethionyl-leucyl-phenylalanine

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N-Formyl-Met-Leu-Phe (FMLP), at concentrations as low as 5 nM, caused an increase in intracellular uridine pools in dimethyl sulphoxide (Me_2SO)-differentiated HL-60 cells. Intracellular uridine pools were elevated rapidly and reached a maximum within 10 min of exposure to 10 μM FMLP, followed by a gradual decline. This enhancement by FMLP was a consequence of a 3-fold increase in the $V_{max.}$ of pertussis-toxin-sensitive Na^+-dependent uridine transport system, with no change in the apparent K_m. K_m values of 2.67 ± 0.45 and 3.85 ± 0.52 μM and $V_{max.}$ values of 0.046 ± 0.017 and 0.125 ± 0.020 μM/s were obtained for untreated and FMLP-treated Me_2SO-differentiated cells respectively. The effect of FMLP on the Na^+-dependent transport of uridine in Me_2SO-differentiated HL-60 cells was specific, as the facilitated transport of uridine was unaffected. Furthermore, this phenomenon was not observed in undifferentiated, phorbol 12-myristate 13-acetate (PMA)-differentiated or pertussis-toxin-treated Me_2SO-differentiated HL-60 cells. Removal of extracellular Ca^{2+} with EGTA abolished the FMLP enhancement of uridine transport in a reversible manner, suggesting the involvement of Ca^{2+}. However, the Ca^{2+} ionophore A23187 only partially mimicked the effect of FMLP. Similarly, with PMA the transport was sub-optimally enhanced, but a full activation was observed in cells treated with both A23187 and PMA. These findings suggest that activation of the Na^+-dependent uridine transporter by FMLP in Me_2SO-differentiated HL-60 cells involves a pertussis-toxin-sensitive G-protein with a bifurcating signal-transduction pathway.

INTRODUCTION

Induction of granulocytic differentiation of HL-60 human promyelocytic leukaemia cells activates a high-affinity Na^+-dependent concentrative uridine transport system that is sensitive to pertussis toxin (Sokoloski et al., 1991). Consequently, relatively large pools of uridine are formed inside these cells, a phenomenon that precedes the appearance of measurable markers of the mature state (Lee et al., 1990). This transport system is present but functionally inactive in undifferentiated HL-60 cells (Lee et al., 1990). The accumulation of huge intracellular concentrations of uridine has not been observed in undifferentiated HL-60 cells or in a variety of tumour cell lines (C.-W. Lee and R. E. Handschumacher, unpublished work), but it has in several normal mouse tissues (Darnowski and Handschumacher, 1986).

It is possible that activation of the G-protein in mature HL-60 granulocytes may result in a further stimulation of the activated Na^+-dependent uridine transport activity. Therefore we have chosen to determine the effects of *N*-formyl-Met-Leu-Phe (FMLP) on the Na^+-dependent transport of uridine in dimethyl sulphoxide (Me_2SO)-differentiated HL-60 cells. FMLP is a well-characterized bacterial chemotactic peptide and a phagocyte activator (Schiffmann et al., 1975; Showell et al., 1976; Marasco et al., 1984) in normal neutrophils and in Me_2SO-differentiated HL-60 cells (Williams et al., 1977; Niedel et al., 1980). The formyl-peptide receptor is coupled to phospholipase C, leading to the production of diacylglycerol and inositol triphosphate ($Ins P_3$) (Dougherty et al., 1984). Diacylglycerol and $Ins P_3$ then serve as putative second messengers for protein kinase C (PKC) activation and intracellular Ca^{2+} mobilization respectively (Virgilio et al., 1985; Pozzan et al., 1983). ADP-ribosylation of a G-protein induced by pertussis toxin prevents signal transduction by this pathway.

The binding of the formyl-peptide to its receptor stimulates chemotaxis, superoxide production, secretion of cell granules and cellular aggregation (Brandt et al., 1985; Krause et al., 1985; Rossi, 1986; Snyderman et al., 1986; Cowen et al., 1990). However, since little is known about the effect of FMLP on nucleoside transport activity, the present work was designed to investigate the mechanism by which FMLP enhanced uridine transport in differentiated HL-60 leukaemia cells. A preliminary account of some of the work presented here has been published in abstract form (Goh et al., 1992).

EXPERIMENTAL

Materials

[5-^{3}H]Uridine (36.5 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). FMLP, Me_2SO, phorbol 12-myristate 13-acetate (PMA) and A23187 were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ^3H_2O (89 μCi/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.). All other reagents were of analytical grade.

Cell culture

Human HL-60 promyelocytic leukaemia cells were cultured as described by Lee et al. (1991) with slight modification. Briefly, cells were maintained in continuous culture in RPMI 1640

Abbreviations used: FMLP, *N*-formylmethionyl-leucyl-phenylalanine; PMA, phorbol 12-myristate 13-acetate; Me_2SO, dimethyl sulphoxide; PKC, protein kinase C.

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medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% (v/v) heat-inactivated (50 °C for 30 min) fetal-bovine serum (Gibco) plus 50 units/ml penicillin and 50 μg/ml streptomycin (Gibco) until passage 65. Cellular differentiation was induced by suspending cells at an initial density of 5×10^4 cells/ml in 75 cm^2 plastic tissue-culture flasks in the presence of 1.0% Me_2SO for 7 days. A Coulter particle counter (Coulter Electronics, Luton, Beds., U.K.) was employed for the determination of cell numbers. The functional maturity of Me_2SO-induced HL-60 cells was assessed by the Nitro Blue Tetrazolium dye-reduction assay (Sokoloski et al., 1986).

Measurement of uridine transport

Me_2SO-differentiated cells were harvested and prepared as described previously (Lee et al., 1991). The uptake of [^{3}H]uridine (5 μM) was initiated by mixing 70 μl of cell suspension containing $(2–3) \times 10^6$ cells, in the presence or absence of 20 μM dipyridamole, with 140 μl of [^{3}H]uridine with or without Na^+, in a 1.5 ml microfuge tube at 22–24 °C. Detailed composition of the final incubation medium is provided in each Figure legend. The reactions were stopped by the 'oil-stop' method as described previously (Lee et al., 1991). For experimental purposes, Na^+-dependent uridine transport is herein defined as the difference between the intracellular concentration of [^{3}H]uridine in cells treated with 20 μM dipyridamole to block facilitated diffusion, in the presence or absence (choline replacement) of Na^+. Facilitated transport of uridine is defined as the difference between the intracellular concentration of [^{3}H]uridine in cells suspended in Na^+-free medium (choline replacement) treated with or without 20 μM dipyridamole. Approximate initial rates of Na^+-dependent and facilitated uridine transport were determined by using a 20 s time interval. This time point was chosen as it fell within the initial linearity of the transport curve, as shown in our control experiments, and no metabolites other than uridine were detected as analysed by h.p.l.c. (results not shown). Measurements of uridine pools were otherwise conducted after 5 min of uptake. The intracellular volume was estimated in all experiments by using ^3H_2O to determine total water space and 'time-zero' values of [^{3}H]uridine transport as extracellular space. 'Time-zero' values for transport, attributed to radioactivity trapped in the cell pellet, were determined by centrifuging 20 μl of the cell suspension through a layer of radioactive substrate (40 μl), which in turn was layered over the oil in an 'oil-stop' tube. The values obtained were similar to those determined by using [^{14}C]inulin as a measurement of extracellular space (results not shown). Kinetic constants for Na^+-dependent uridine transport were assessed by use of the non-linear least-squares fit of the Michaelis–Menten equation using the ENZFIT computer program (Leatherbarrow, 1987).

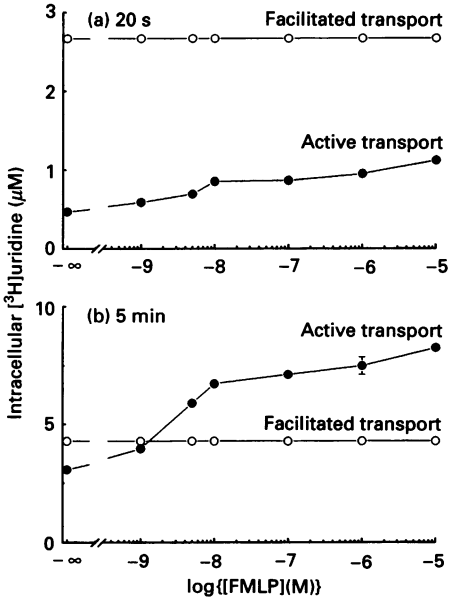

Figure 1 Effects of various concentrations of FMLP on the increase in Na^+-dependent uridine transport

Na^+-dependent (●) and facilitated (○) transport of 5 μM [^{3}H]uridine at uptake intervals of 20 s (**a**) or 5 min (**b**) was determined in Me_2SO-differentiated HL-60 cells treated with 0–10 μM FMLP for 15 min. The amount of intracellular free [^{3}H]uridine was determined by h.p.l.c. as described in the Experimental section. The data shown are representative of at least three separate experiments, and the values presented are means ± S.E.M. of triplicate determinations; where absent, the S.E.M. is smaller than the symbol employed.

Measurement of intracellular free uridine

The trichloroacetic acid-digested cell pellet from each 'oil-stop' tube was extracted with an equal volume of 1 M trioctylamine in Freon (Darnowski and Handschumacher, 1986). The form of the ^{3}H radioactivity in the cell pellet extract was assessed by h.p.l.c. using a Waters Nova-Pack C_{18} reverse-phase column. The mobile phase consisted of 10 mM H_3PO_4 and 30 μM heptanesulphonic acid (pH 3.3), with a flow rate of 1 ml/min at 22–24 °C. Under these conditions, the following retention times were observed: void volume, 2 min; uracil nucleotides, 4–5 min; uracil, 6 min; and uridine, 10 min.

RESULTS

Concentration–response of FMLP-induced increase in Na^+-dependent uridine transport

Me_2SO-differentiated HL-60 cells were exposed to various concentrations of FMLP (0–10 μM) for 15 min, inclusive of time taken for transport. Subsequently, both facilitated and Na^+-dependent transport as well as intracellular accumulation of [^{3}H]uridine were measured. Figure 1(a) illustrates that FMLP at concentrations as low as 5 nM enhanced the Na^+-dependent transport of uridine. With 10 μM FMLP, the Na^+-dependent uridine transport activity was 2.4-fold (1.120 μM/20 s) above its basal level (0.469 μM/20 s). This potentiation by FMLP of the Na^+-dependent transport of uridine resulted in a similar 2.7-fold (8.249 μM/5 min) elevation in the intracellular concentration of [^{3}H]uridine over that of the basal level (3.073 μM/5 min), as shown in Figure 1(b). The increase in Na^+-dependent uridine transport produced by FMLP was specific, as facilitated transport and the accumulation of intracellular [^{3}H]uridine through this mechanism were not affected by the chemotactic peptide (Figures 1a and 1b). Furthermore, the stimulatory effect of FMLP was not observed in undifferentiated, PMA-differentiated or pertussis-toxin-treated Me_2SO-differentiated HL-60 cells (results not shown).

Time course of FMLP-induced increase in Na^+-dependent uridine transport

Figure 2(a) shows that the enhancement of Na^+-dependent transport of [^{3}H]uridine by FMLP was rapid and transient. The transport activity reached a peak at about 10 min, followed by a gradual decline to near basal level. The increase in the rate of uptake of uridine was greater with 10 μM FMLP. The levels of accumulated intracellular [^{3}H]uridine pools showed a similar time-response to FMLP (Figure 2b).

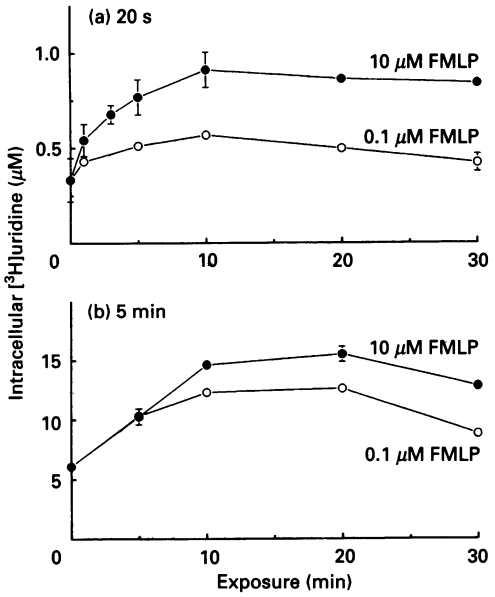

Figure 2 Time course of the FMLP-induced increase in Na^+-dependent uridine transport

Na^+-dependent transport of 5 μM [^{3}H]uridine measured at uptake intervals of 20 s (**a**) or 5 min (**b**) were determined in Me_2SO-differentiated HL-60 cells treated with either 10 μM (●) or 0.1 μM (○) FMLP for 0–30 min. The amount of intracellular free [^{3}H]uridine was determined by h.p.l.c. as described in the Experimental section. The data shown are representative of three or more separate experiments, and each value represents the mean ± S.E.M. of triplicate determinations; where absent, the S.E.M. is smaller than the symbol employed.

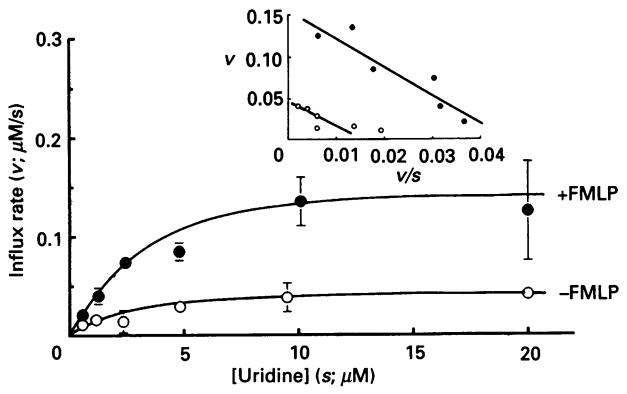

Figure 3 Effects of FMLP on the kinetics of Na^+-dependent uridine transport

Concentration-dependence of Na^+-dependent [^{3}H]uridine transport in Me_2SO-differentiated HL-60 cells treated with (●) or without (○) 10 μM FMLP for 10 min was measured at 15 s uptake intervals. The data shown were corrected for the linear component of [^{3}H]uridine transport determined in the Na^+-free (choline replacement) dipyridamole-containing medium, and the values shown are means ± S.E.M. of triplicate determinations; where absent, the S.E.M. is smaller than the symbol employed. The kinetic constants for the data presented are K_m values of 3.179 ± 0.088 and 3.296 ± 1.330 μM, and $V_{max.}$ values of 0.156 ± 0.015 and 0.048 ± 0.007 μM/s for FMLP-treated and untreated cells, respectively.

Effects of FMLP on the kinetics of Na^+-dependent uridine transport

Figure 3 shows the saturable transport component in differentiated HL-60 cells treated with or without 10 μM FMLP for 10 min, after subtraction of the linear component measured in the absence of Na^+ (choline replacement) and in the presence of 20 μM dipyridamole to inhibit facilitated diffusion. These kinetic studies show a 3-fold increase in the $V_{max.}$ of transport in FMLP-treated cells (0.125 ± 0.020 μM/s; $n = 7$), compared with that of untreated cells (0.046 ± 0.017 μM/s; $n = 7$), with little change in the affinity of the transporter for uridine [apparent K_m values of 3.85 ± 0.52 and 2.67 ± 0.45 μM ($n = 7$) for FMLP-treated and untreated cells respectively]. Eadie–Hofstee plots of these data revealed a single high-affinity transport component in both FMLP-treated and untreated Me_2SO-differentiated HL-60 cells (Figure 3, inset).

Effects of Ca^{2+} on the FMLP-induced increase in Na^+-dependent uridine transport

Control experiments demonstrated that FMLP at concentrations as low as 5 nM elicits a transient increase in the intracellular levels of Ca^{2+} in Me_2SO-differentiated HL-60 cells. Cytosolic Ca^{2+} reaches its maximal concentration rapidly within 10–30 s, followed by a gradual return to its basal level within 5 min of exposure to FMLP (results not shown). The rapidity and potency of FMLP in eliciting an increase in the intracellular levels of both

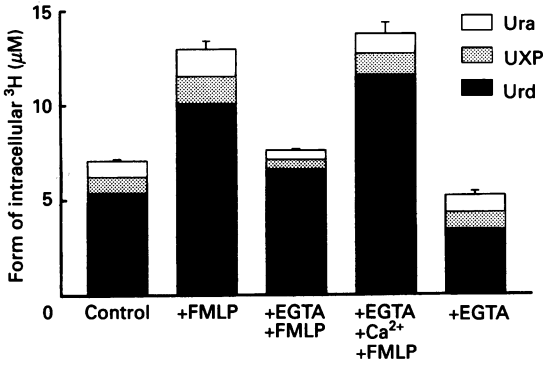

Figure 4 Requirement for Ca^{2+} in the FMLP-induced increase in intracellular uridine

The effects of Ca^{2+} depletion on the Na^+-dependent accumulation of intracellular [^{3}H]uridine in Me_2SO-differentiated HL-60 cells were determined at 5 min uptake intervals. Cells suspended in Ca^{2+}-containing medium were treated with or without 10 μM FMLP for 10 min. In some instances, extracellular Ca^{2+} was chelated with 1.5 mM EGTA, and in others 1.4 mM Ca^{2+} was added before FMLP exposure. The form of the intracellular radioactivity was determined by h.p.l.c. as described in the Experimental section. The data shown are representative of four individual experiments; values are means ± S.E.M. of triplicate determinations. Abbreviations: Ura, uracil; Urd, uridine; UXP, uracil nucleotides.

cytosolic Ca^{2+} and uridine suggest a common mechanism for these two biochemical events.

To evaluate this possibility, 1.4 mM EGTA was added before the exposure of Me_2SO-differentiated HL-60 cells to FMLP. Figure 4 shows that EGTA effectively abolished that portion of the Na^+-dependent accumulation of [^{3}H]uridine induced by 10 μM FMLP, but had little impact on existing [^{3}H]uridine pools. Re-introduction of Ca^{2+} into the incubation medium

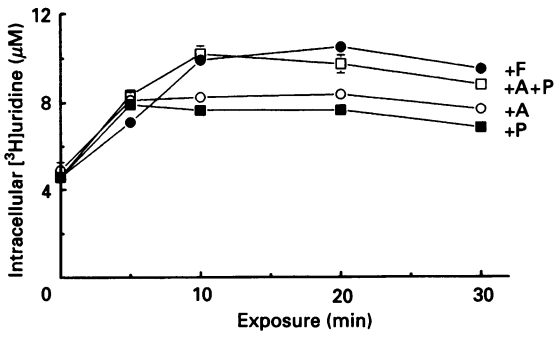

Figure 5 Effects of A23187 and PMA on intracellular levels of uridine

Na^+-dependent accumulation of 5 μM [^{3}H]uridine was determined in Me_2SO-differentiated HL-60 cells treated with 10 μM FMLP (F; ●), 5 μM A23187 (A; ○), 200 ng/ml PMA (P; ■), or both A23187 and PMA (A + P; □), at an uptake interval of 5 min. The amount of intracellular free [^{3}H]uridine was determined by h.p.l.c. as described in the Experimental section. The data shown are representative of three individual experiments, and values are means ± S.E.M. of triplicate determinations; where absent, the S.E.M. is smaller than the symbol employed.

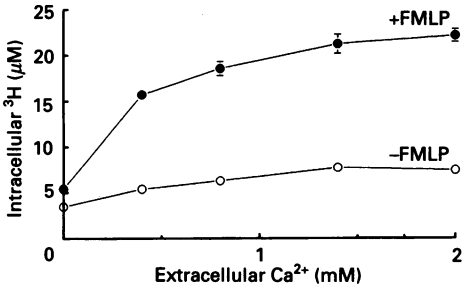

Figure 6 Concentration–response of Na^+-dependent uridine transport to extracellular Ca^{2+}

Na^+-dependent transport (20 s) of 5 μM [^{3}H]uridine was measured in Me_2SO-differentiated HL-60 cells treated with (●) or without (○) 10 μM FMLP for 10 min in the presence of 0–2 mM extracellular Ca^{2+}. The data shown are representative of four separate experiments, and values are means ± S.E.M. of triplicate determinations; where absent, the S.E.M. is smaller than the symbol employed.

(total $[Ca^{2+}]_{out}$ = 2.8 mM) completely reversed the inhibitory action of EGTA. These effects were specific, since the same amount of EGTA did not affect the simple diffusion or facilitated transport of [^{3}H]uridine, a measure of membrane integrity (results not shown).

Effects of A23187 and PMA on Na^+-dependent accumulation of uridine

After 30 min of exposure of 5 μM of the Ca^{2+} ionophore A23187, a 1.6-fold increase (from 4.89 to 7.69 μM/5 min) in the intracellular concentration of [^{3}H]uridine occurred in control cells that were not exposed to FMLP (Figure 5). However, the extra amount of [^{3}H]uridine accumulated was only about 50 % of that in cells treated with 10 μM FMLP (10.49 μM/5 min). Similarly, with 200 ng/ml of a PKC activator, PMA, the accumulation of intracellular [^{3}H]uridine was also augmented. It reached a peak after 5 min of exposure (7.92 μM/5 min) and was lower than that in FMLP-treated cells. However, a combination of A23187 and PMA evoked a response as great as that of the chemotactic peptide. Exposure of FMLP-treated cells to the Ca^{2+} ionophore or to PMA did not cause a further increase in the concentration of intracellular [^{3}H]uridine (results not shown).

Effects of various concentrations of extracellular Ca^{2+} on Na^+-dependent accumulation of uridine

Figure 6 shows that the Na^+-dependent accumulation of uridine in both FMLP-treated and untreated Me_2SO-differentiated HL-60 cells responded to extracellular Ca^{2+} in a concentration-dependent manner. However, the requirement for Ca^{2+} was more pronounced in FMLP-treated cells than in untreated cells, with K_{Ca} values of 0.376 ± 0.029 mM and 0.815 ± 0.244 mM respectively.

DISCUSSION

In the present study, we have demonstrated that FMLP is capable of causing a rapid but transient increase in the pertussis-toxin-sensitive Na^+-dependent transport of uridine in Me_2SO-differentiated HL-60 cells. This phenomenon was not observed in undifferentiated cells, or in cells that matured along the monocytic route after exposure to PMA. The former can be explained by the lack of FMLP receptors on the cell surface (Niedel et al., 1980) and the functional inactivity of the transporter (Lee et al., 1990). However, the PMA-differentiated cells possess an activated transport system with characteristics similar to those of Me_2SO-differentiated HL-60 cells (Lee et al., 1991). Thus the unresponsiveness of this system may be due to an incompletely developed signal-transduction pathway involving G-protein during differentiation.

Concentration-dependent changes in Na^+-dependent uridine transport were also found to occur upon exposure of cells to FMLP. With nanomolar concentrations of FMLP, the transport activity accelerated rapidly, followed by a plateau at FMLP levels between 10 nM and 1 μM. Further acceleration to a new level of uridine transport occurs in the presence of 10 μM FMLP. It has been reported that at relatively low concentrations of FMLP (nanomolar range) only chemotactic responses are elicited from phagocytes, whereas at higher concentrations of FMLP (micromolar range), cytotoxic responses are expressed (Snyderman and Pike, 1984; Perez et al., 1989). Thus it appears that different intracellular levels of uridine, elicited by different concentrations of FMLP, may be associated with cellular requirements for different responses.

The mechanism behind the potentiation of the Na^+-dependent uridine transport by FMLP has yet to be elucidated. However, studies of the transport kinetics suggest that the enhancement in uridine transport produced by FMLP is a consequence of an increase in the number of Na^+-dependent uridine transporters, but the apparent transport affinity for uridine remains unchanged. The source of the additional Na^+-dependent uridine transporter molecules is unknown. It is possible that some cryptic transporter molecules exist on the membrane surface and are activated by FMLP ligand–receptor complexes. Alternatively, transporter molecules may have been recruited from an intracellular source by exocytosis, as the ability of FMLP to induce membrane degranulation and addition in human neutrophils is well documented (Lacal et al., 1988; Hoffstein et al., 1982).

It is conceivable that formation of the FMLP ligand–receptor complex triggered the transient increase in intracellular uridine pools concurrent with and in a manner analogous to that occurring with cytosolic Ca^{2+}. In this regard, the removal of Ca^{2+} from the incubation medium by EGTA should effectively abolish

the effects of FMLP, and be completely reversed when Ca^{2+} was replenished. This is in agreement with our finding, suggesting the involvement of Ca^{2+} in the activation of the Na^+-dependent transport of uridine. However, in cells that were not exposed to FMLP but permeabilized to Ca^{2+} with the Ca^{2+} ionophore A23187, intracellular uridine pools were only partially elevated. Thus, although Ca^{2+} may be involved, activation of the Na^+-dependent uridine transport system may require other components that are not activated by A23187. It is unlikely that the unregulated rise in cytosolic Ca^{2+} induced by A23187 is somehow inhibitory, as no such inhibition by the ionophore was observed in FMLP-treated cells.

We have demonstrated that the other signal component involved in the activation of this transporter could be a PKC. In the presence of the PKC activator PMA, the enhancement in transport activity was similar to that obtained with A23187. However, full activation of the Na^+-dependent uridine transport occurred when cells were treated with both A23187 and PMA. Hence, activation of the Na^+-dependent uridine transporter in Me_2SO-differentiated HL-60 cells involves a pertussis-toxin-sensitive G-protein with a bifurcating signal-transduction pathway. These actions may eventually lead to the onset of several phosphorylation events, and consequently the activation of the Na^+-dependent uridine transporter. Details of the entire activation pathway have yet to be elucidated.

The requirement for Ca^{2+} in the action of FMLP on Na^+-dependent uridine transport in differentiated HL-60 cells is evident. However, the precise role of Ca^{2+} in this process is complicated by the observation that existing transporters are also dependent on the extracellular concentration of Ca^{2+}, although to a smaller extent. This is apparent in cells not treated with FMLP (Figure 6). Nevertheless, the present study shows that the Na^+-dependent uridine transporter can be activated in granulocyte-like cells by an agonist of G-protein, FMLP. The elevation of intracellular uridine pools produced by FMLP in Me_2SO-differentiated HL-60 cells may be reproduced by other substances that are known to activate neutrophils. Such a possibility is currently under investigation.

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