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Inhibition by pertussis toxin of the activation of Na^+-dependent uridine transport in dimethyl-sulphoxide-induced HL-60 leukaemia cells

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The effects of pertussis toxin on the Na^+-dependent transport of uridine were studied in HL-60 leukaemia cells induced to differentiate along the granulocytic or monocytic pathways by dimethyl sulphoxide (DMSO) or phorbol 12-myristate 13-acetate (PMA) respectively. Pertussis toxin at 50 ng/ml completely inhibited the activation of Na^+-dependent uridine transport and consequently prevented the formation of intracellular pools of free uridine which occurs in HL-60 cells induced to differentiate by DMSO. The inhibition of Na^+-dependent uridine transport by pertussis toxin in cells exposed to DMSO was associated with a 14-fold decrease in affinity, with no change in $V_{max.}$. Pertussis toxin, however, had no effect on Na^+-dependent uridine transport in PMA-induced HL-60 cells. Furthermore, 500 ng of cholera toxin/ml had no effect on the Na^+-dependent uptake of uridine in DMSO-treated HL-60 cells. These results suggest that the activation of the Na^+-dependent transport of uridine in HL-60 cells induced to differentiate along the granulocytic pathway by DMSO is coupled to a pertussis-toxin-sensitive guanine-nucleotide binding protein (G-protein).

INTRODUCTION

The entry of uridine and other nucleosides into mammalian cells occurs via saturable transporter mechanisms that have been widely studied. These transporters are generally considered in two categories; facilitated diffusion [1,2] and Na^+-dependent active transport [3–7]. The facilitated diffusion mechanisms are predominantly sensitive to inhibition by nitrobenzylthioinosine (NBMPR) [8,9], but insensitive forms of these transporters have been found [10–12]. The Na^+-dependent process had been reported to exist in polarized cell types such as renal and intestinal epithelial cells that have brush borders [5,13,14]. Based upon multisubstrate transport and inhibition studies, it has been suggested that at least two forms of the Na^+-dependent driven transporter may exist [15]. These epithelial cells also express the facilitated diffusion system, predominantly in the basolateral membrane [13]. Previous findings in our laboratory have documented the presence of the Na^+-dependent transporter in non-polarized splenocytes and thymocytes [7,16–18]. Subsequently, minimal amounts of the Na^+-dependent mechanism have been detected in some lymphoid neoplastic cell lines [12,19]; however, in these neoplastic cells the activity is insufficient to generate a concentration gradient of uridine. To our knowledge, these nucleoside transport mechanisms do not normally result in active concentration of purine nucleosides in any normal or neoplastic tissues. In contrast, uridine achieves normal tissue concentrations that range from 5 to 10 times that in plasma, while in neoplastic cell lines and in a limited number of primary neoplasms, concentrations of this nucleoside approximate that in plasma [16].

We have shown that the entry of uridine into undifferentiated HL-60 cells occurs predominantly by the NBMPR-sensitive facilitated diffusion mechanism [20,21]. A very minor second component of uridine flux that is Na^+-dependent exists in undifferentiated HL-60 cells. When HL-60 cells are induced to differentiate into mature granulocyte-like cells by dimethyl sulphoxide (DMSO) [20], or to monocyte-like cells by phorbol 12-myristate 13-acetate (PMA) [21], a decrease in facilitated diffusion as well as in uridine metabolism is observed. At the same time, however, the activity of the Na^+-dependent uridine flux is markedly enhanced in differentiated HL-60 cells. The sum of these changes results in the generation of intracellular uridine concentrations that are 10–15-fold higher than in the medium at a physiological concentration of 1 μM-uridine. This gradient of uridine is not present in undifferentiated cells. The increase in Na^+-dependent uptake activity that occurs in HL-60 cells differentiated by DMSO is attributable to a 28-fold increase in transporter affinity for uridine over that in undifferentiated HL-60 cells, with no apparent change in $V_{max.}$. These observations suggest that the Na^+-dependent uridine transporter may be regulated by an unknown factor(s) that is either absent or functionally uncoupled in undifferentiated HL-60 leukaemia cells.

Recent studies show that guanine-nucleotide-binding proteins (G-proteins) regulate many cellular processes, including receptor-mediated signal transduction [22–24] and stimulation of K^+ channels [25] and certain Ca^{2+} channels [26], and possibly some amiloride-sensitive Na^+ channels [27]. G-proteins also participate in a variety of intracellular processes, including membrane transport, exocytosis and secretion [28–30]. Thus it is conceivable that a G-protein may be involved in the activation of the Na^+-dependent uridine transporter in DMSO-induced HL-60 cells.

In this report, we demonstrate that pertussis toxin abolishes the activation of Na^+-dependent uridine flux and consequently the formation of a uridine concentration gradient in HL-60 cells induced to differentiate along the granulocytic pathway by DMSO. The inhibitory effect of pertussis toxin on Na^+-dependent uridine transport was reflected in a marked reversion in the affinity of this carrier for uridine to values comparable with those present in undifferentiated HL-60 cells. Pertussis toxin, however, had no effect on Na^+-dependent uridine uptake in HL-60 leukaemia cells induced to differentiate to monocyte-like cells by

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; DMSO, dimethyl sulphoxide; NBMPR, nitrobenzylthioinosine; G-protein, guanine-nucleotide-binding protein; NBT, Nitro Blue Tetrazolium.

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PMA; this result suggests that the phorbol ester may cause G-protein-independent changes in transporter activity, presumably due to the activity of protein kinase C [31]. The finding that the Na^+-dependent uridine transport system is functionally coupled to a pertussis-toxin-sensitive G-protein provides a useful model to investigate further the regulation of this transporter, as well as to determine the importance of the uridine transporter to the differentiated phenotype.

MATERIALS AND METHODS

Chemicals

[5-^{3}H]Uridine (20 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, CA, U.S.A.). ^3H_2O (18 μCi/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.) and [^{14}C]inulin (10 mCi/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.). DMSO, cholera toxin and pertussis toxin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of analytical grade.

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 nitrogen, and were maintained in continuous culture in RPMI 1640 medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 15% (v/v) heat-inactivated (50 °C for 30 min) fetal bovine serum (Gibco), 50 units of penicillin/ml and 50 μg of streptomycin/ml (Sigma) until passage 65. Cell stocks were routinely monitored for mycoplasma contamination by the gene probe method (Gen Probe Inc., San Diego, CA, U.S.A.).

Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in air. Cellular differentiation was induced by suspending cells at an initial density of 2×10^5 cells/ml in 175 cm^2 Corning plastic tissue culture flasks in the presence of inducing agent for 6 days. Cell numbers were determined with a Coulter particle counter equipped with a Channelizer (Coulter Electronics, Hialeah, FL, U.S.A.).

Cellular differentiation

The capacity of HL-60 leukaemia cells to undergo functional maturation was assessed by Nitro Blue Tetrazolium (NBT) dye reduction [32]. Approx. 2×10^6 cells were collected by centrifugation and resuspended in 1.0 ml of RPMI 1640 complete medium containing 0.1% NBT and 1.0 μg of PMA/ml. The cell suspension was incubated at 37 °C for 30 min, and the percentage of cells containing blue–black formazan deposits, indicative of a PMA-stimulated respiratory burst, was determined by microscopic analysis.

Transport studies

To prepare cells for transport studies, a cell suspension was centrifuged at 1000 *g* for 5 min in a Sorvall GLC-4 centrifuge (Sorvall Instruments, Wilmington, DE, U.S.A.). The resulting cell pellet was washed with 50 ml of Na^+-free (choline replacement) Hanks' balanced salt medium plus 5.5 mM-D-glucose and 4 mM-Hepes buffer (pH 7.4), and centrifuged at 1000 *g* for 5 min. The resulting 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 5 μM-[^{3}H]uridine was initiated by mixing 30 μl of the cell suspension, containing 2×10^6 cells, preincubated with or without 10 μM-NBMPR for 5 min, with 60 μl of radioactive substrate in a 1.5 ml Eppendorf Microfuge tube at 22–24 °C. Uptake of uridine was measured using the 'oil-stop method' previously described [21]. Na^+-dependent uridine uptake was operationally defined as the difference between the intracellular content of [^{3}H]uridine in cells treated with 10 μM-NBMPR to block facilitated diffusion in the presence and the absence (choline replacement) of Na^+. Facilitated diffusion was measured as the influx of [^{3}H]uridine in the absence of Na^+ (choline replacement), corrected for the influx in the absence of Na^+ and the presence of NBMPR. The approximate initial rate of Na^+-dependent uridine transport was determined using a 6 s time interval. Control experiments showed that no significant metabolism of uridine occurred within the first 15 s of uptake. 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 (40 μl) containing radioactive uridine layered over the oil in an oil-stop tube. These values were subtracted from measurements of uridine uptake by HL-60 cells. The intracellular volume of HL-60 cells was calculated in all experiments, using ^3H_2O to determine total water space and [^{14}C]inulin to calculate extracellular space [7]. Kinetic constants for Na^+-dependent uridine transport were determined by non-linear least-squares fit of the Michaelis–Menten equation using the computer program ENZFITTER [33].

Intracellular free uridine

Cells were incubated with 5 μM-[^{3}H]uridine at 22 °C for 15 and 300 s intervals, and 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-trioctylamine in Freon [21]. The form of ^{3}H radioactivity from uridine in the cell pellet was assessed by h.p.l.c. using a C_{18} reverse-phase column. The mobile phase consisted of 10 mM-phosphoric acid and 30 μM-heptane/sulphonic 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; uridine, 16 min.

RESULTS

To develop an understanding of the functional controls on the Na^+-dependent transporter induced in HL-60 cells by DMSO, the effects of bacterial toxins with relatively specific targets were investigated. Initial experiments assessed the overall utilization of uridine during extended time periods to establish the physiological significance of changes in transport mechanisms. Total uridine uptake in undifferentiated HL-60 cells (Fig. 1*a*) is rapid, and associated with extensive nucleotide formation [20,21]. The primary mode of entry is via a non-concentrative process, since intracellular free uridine concentrations do not exceed those in the medium [20] and uptake is virtually abolished by the presence of NBMPR. In addition to facilitated diffusion, a very minimal level of Na^+-dependent uridine flux can be detected in undifferentiated HL-60 cells (Fig. 1*a*). The Na^+-dependent uptake was defined as that portion of uridine utilization detected in the presence of sodium and NBMPR after correction for that found in Na^+-free (choline replacement) NBMPR-containing medium. When HL-60 cells were induced to differentiate along the granulocytic pathway by 1.5% DMSO, a marked decrease in uptake dependent on the facilitated diffusion of uridine was observed (Fig. 1*b*); this effect corresponded to a comparable decrease in NBMPR-binding sites and specifically documented transport by the facilitated diffusion transporter [21]. However, the induction of differentiation by DMSO was also accompanied by a substantial increase in the rate of uridine uptake by a Na^+-dependent process. The increase in the Na^+-dependent process

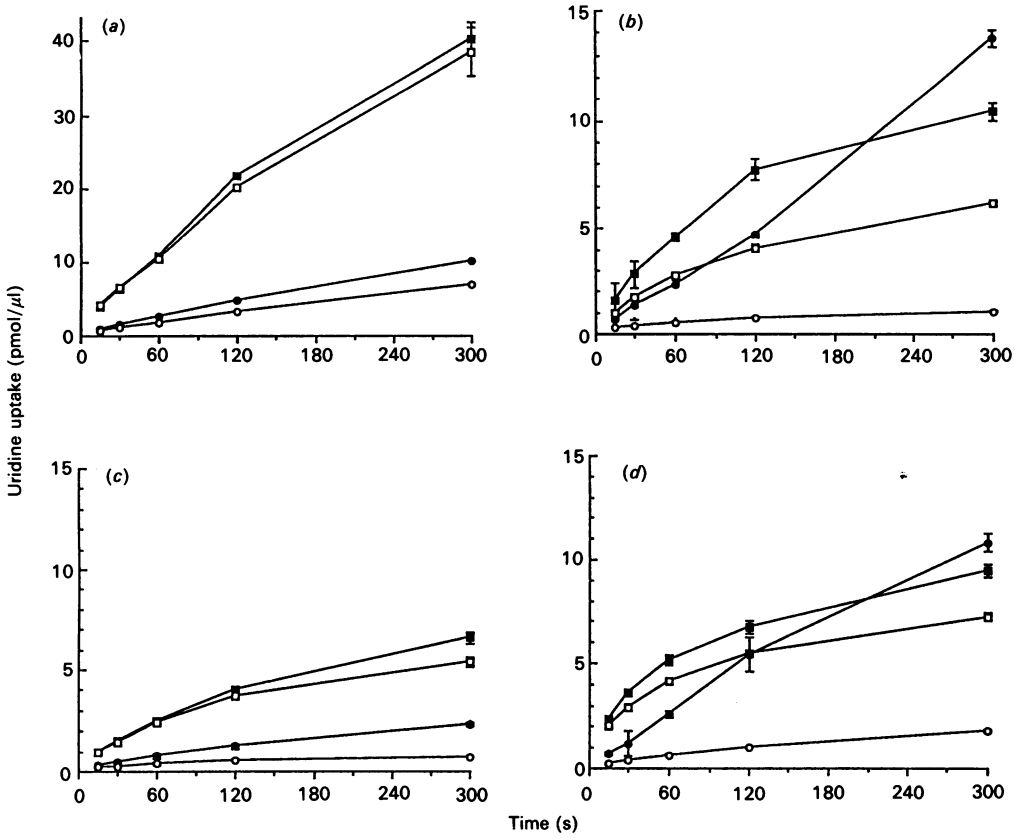

Fig. 1. Time course of uridine uptake in HL-60 cells induced by DMSO to differentiate to granulocyte-like cells

Cells at an initial concentration of 2×10^5 cells/ml in RPMI 1640 medium were incubated in the absence (*a*) or presence (*b*, *c*, *d*) of 1.3% DMSO for 6 days. During the final 18 h of incubation, cells were treated with vehicle (*a*, *b*), 5.0 ng of pertussis toxin/ml (*c*) or 500 ng of cholera toxin/ml (*d*) for 18 h. Cells were then harvested, washed and incubated with (●, ○) or without (■, □) 10 μM-NBMPR for 5 min before exposure to [^{3}H]uridine (5 μM final concentration) in the presence (●, ■) or absence (○, □) of Na^+ (choline replacement). At appropriate time intervals, uptake was terminated by the oil-stop method described in the Materials and methods section. Data are from an experiment representative of at least three experiments. Values are the means ± S.E.M. of triplicate determinations; where absent, the S.E.M. was smaller than the symbol.

was further enhanced when the efflux of uridine by facilitated diffusion was blocked by NBMPR. Under these conditions, the capacity of differentiated HL-60 cells induced by DMSO to utilize exogenous uridine was eliminated by the absence of Na^+ (Fig. 1*b*). Pertussis toxin (5.0 ng/ml) present during the final 18 h of the 6th day of exposure to DMSO completely abolished the uptake of uridine by the Na^+-dependent process (Fig. 1*c*). Under identical conditions, cholera toxin had no effect on uridine utilization (Fig. 1*d*).

To relate the toxin effects directly to the Na^+ transporter, all subsequent experiments measured specific transport and intracellular concentrations of free uridine as determined by h.p.l.c. The initial rate of transport was linear over 15–20 s and is presented as total flux at 15 s. The facilitated diffusion component was totally insensitive to pertussis toxin and somewhat greater than the Na^+-dependent system. The Na^+-dependent system, however, produces a large gradient of free uridine at subsequent time periods (5 min) and is highly sensitive to pertussis toxin, with an IC_{50} (concn. causing 50% inhibition) of < 1 ng/ml.

Treatment of differentiated HL-60 cells induced by the polar solvent (day 6) with either 50 ng of pertussis toxin/ml or 500 ng of cholera toxin/ml for 18 h did not significantly affect the expression of NBT-positivity by these cells, a marker of the mature phenotype (results not shown). To determine the sensitivity of uridine transport to pertussis toxin, DMSO-differentiated HL-60 cells were exposed to various concentrations of pertussis toxin for 18 h. Concentrations of pertussis toxin as low as 1 ng/ml inhibited Na^+-dependent uridine transport (Fig. 2*a*) and consequently interfered with the accumulation of intracellular pools of free uridine (Fig. 2*b*). Maximum inhibition of Na^+-dependent uridine transport was observed at pertussis toxin concentrations of 5 ng/ml. The onset of inhibition by this concentration of pertussis toxin was apparent within 8 h of exposure of the DMSO-induced cells, and was essentially complete within 18 h (Fig. 3*a*); consequently, the accumulation of free uridine gradients within the cell was eliminated by more than 12 h of exposure to pertussis toxin (Fig. 3*b*).

The inhibitory effects of pertussis toxin on the Na^+-dependent uridine transporter in differentiated HL-60 cells induced by DMSO can be attributed to a decrease in transport affinity from a K_m of 1.4 μM for the DMSO-differentiated cells to a K_m of 21.4 ± 4.8 μM in the presence of pertussis toxin. This value is similar to that observed in the undifferentiated cell population (K_m 44 μM). There was no change in the apparent $V_{max.}$ (0.16 ± 0.02 pmol/s per μl). Treatment of differentiated HL-60 cells with 500 ng of cholera toxin/ml had no effect on the activity (Fig. 1*d*) or the K_m and $V_{max.}$ values of the Na^+-dependent uridine transporter (results not shown).

When HL-60 cells were induced by PMA to differentiate along the monocytic pathway, the effects of pertussis toxin on the Na^+-dependent transport system were not significantly affected (Table 1). The decrease in Na^+-dependent uptake caused by 500 ng of

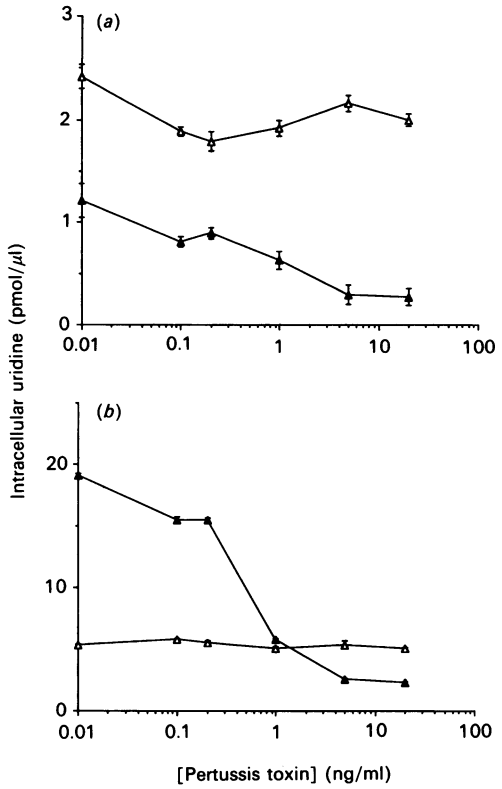

Fig. 2. Effects of pertussis toxin concentration on uridine transport and accumulation of intracellular uridine in HL-60 cells induced by DMSO to differentiate to granulocyte-like cells

HL-60 cells exposed to 1.3% DMSO for 6 days were incubated with various concentrations of pertussis toxin (0–20 ng/ml) for 18 h. Cells were harvested and washed, and [^{3}H]uridine (5 μM final concentration) 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 15 s (*a*) and 5 min (*b*) intervals were measured. Intracellular free [^{3}H]uridine concentrations were determined by h.p.l.c. as described in the Materials and methods section. Data are the means ± S.E.M. from a representative experiment consisting of triplicate samples from three independent experiments.

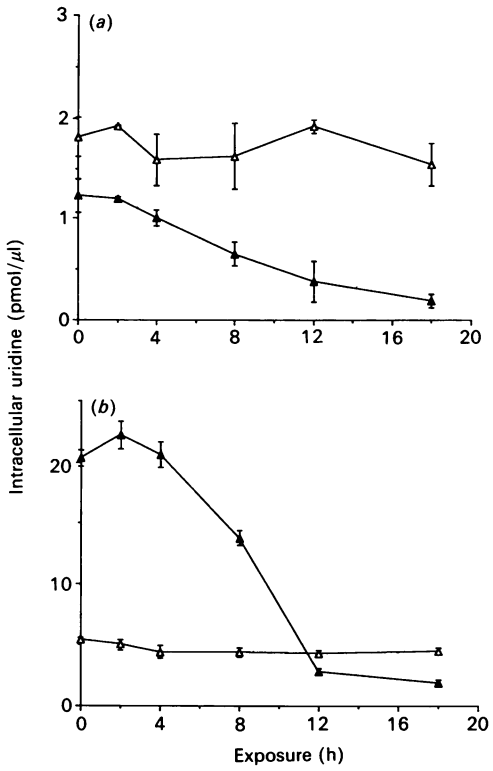

Fig. 3. Effects of the duration of exposure to pertussis toxin on uridine transport and accumulation of intracellular uridine in HL-60 cells induced by DMSO to differentiate to granulocyte-like cells

HL-60 cells exposed to 1.3% DMSO for 6 days were incubated with 5 ng of pertussis toxin/ml for between 0 and 18 h. At the indicated times, cells were harvested and washed, and [^{3}H]uridine (5 μM final concentration) 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 15 s (*a*) and 5 min (*b*) intervals were measured. Intracellular free [^{3}H]uridine concentrations were determined by h.p.l.c. as described in the Materials and methods section. Data are the means ± S.E.M. from a representative experiment consisting of triplicate samples from three independent experiments.

cholera toxin/ml in HL-60 cells induced to differentiate by PMA (Table 1) may reflect the increased cytoxicity that has been observed with this toxin under these conditions (results not shown).

DISCUSSION

This paper presents findings that suggest a role for G-protein-modulated signal transduction systems in the regulation of active nucleoside transport processes. The specific inhibition by pertussis toxin of the Na^+-dependent uptake of uridine is consistent with the lack of an effect on the facilitated diffusion mechanism in HL-60 cells induced to differentiate along the granulocytic pathway by DMSO. Individual G-proteins are distinguished by their characteristic α-subunits, which are responsible for the binding and hydrolysis of GTP, and for activating specific effector functions. The α-subunit also contains the specific site for NAD^+-dependent ADP-ribosylation, which is catalysed by several bacterial toxins, including cholera, diphtheria and pertussis toxins [34]. Pertussis toxin catalyses the ADP-ribosylation of the α-subunits of G_i and G_o [35]. In contrast, cholera toxin is known to ADP-ribosylate the α-subunit of G_s [36]. Each of these α-subunits contains a highly conserved cysteine residue near the

Table 1. Effects of bacterial toxins on uridine transport in HL-60 cells induced to differentiate to monocyte-like cells by PMA

Untreated HL-60 cells or those treated with 200 ng of PMA/ml for 48 h were exposed to 5 ng of pertussis toxin/ml, 500 ng of cholera toxin/ml or vehicle during the final 18 h of incubation. [^{3}H]Uridine (5 μM) uptake by facilitated diffusion (uptake in the absence of Na^+) and by Na^+-dependent transport (uptake in the presence of Na^+ and 10 μM-NBMPR) were measured for 5 min as described in the Materials and methods section. Data are the means ± S.E.M. of three independent experiments, each performed in triplicate.

	Total intracellular ^{3}H radioactivity (pmol/μl)	
	Na^+-dependent transport	Facilitated diffusion
Control (undifferentiated cells)	5.4 ± 0.4	106.2 ± 3.2
PMA (200 ng/ml)	27.7 ± 0.7	13.8 ± 0.3
+ pertussis toxin (5 ng/ml)	30.0 ± 1.0	15.4 ± 0.6
+ cholera toxin (500 ng/ml)	10.0 ± 0.4	11.6 ± 0.3

C-terminus [37]. Since the *C*-terminus is also the receptor-binding region, the inhibition of G-protein activity by bacterial toxins has provided a useful tool to investigate the role of G-proteins in various cellular functions. The G-protein involved appears to be a G_i and/or G_o subunit, since cholera toxin, a potent inhibitor of the activity of the α-subunit of G_s, did not inhibit the Na^+-dependent uridine uptake in DMSO-induced HL-60 cells. Despite the correlative nature of these findings, it is possible that treatment with pertussis toxin resulted in the inhibition of inositol lipid hydrolysis or of the inhibitory arm of adenylate cyclase, which are sensitive to this bacterial toxin [23]. These effects could in turn modulate the uptake of uridine.

The decrease in Na^+-dependent uridine transport activity was attributed to a marked decrease in uridine transport affinity, with no apparent change in $V_{max.}$ Most strikingly, the affinity of the Na^+-dependent transporter for uridine in the presence of pertussis toxin closely resembled the affinity found in undifferentiated HL-60 cells. It is well-documented that the ADP-ribosylation of G-proteins by pertussis toxin interferes with receptor coupling, but does not modify the intrinsic GTPase activity, GTP binding or subunit dissociation [38]. Thus the modulation of Na^+-dependent uridine uptake and the consequent accumulation of intracellular uridine by pertussis toxin in mature HL-60 cells implies that the cellular machinery which regulates the activity of this transport system may be coupled to a G-protein signal transduction pathway similar to that previously described for the production of superoxide by neutrophils [39]. If such a mechanism is operative, it is possible that the Na^+-dependent nucleoside transport system may be functionally coupled to a specific cell surface receptor. One such receptor could be the fMetLeuPhe receptor. Recent evidence has suggested that the formation of an fMetLeuPhe receptor complex in differentiated HL-60 cells results in the activation of a pertussis-toxin-sensitive GTP-binding protein, believed to be G_i2 from the G_i subfamily of regulatory G-proteins [40]. Thus one might be able to enhance Na^+-dependent transporter activity by activating the fMetLeuPhe receptor(s) that communicates through the pertussis-toxin-sensitive G-protein. This in turn would modulate the activity of the transporter.

In summary, we provide preliminary evidence that a pertussis-toxin-sensitive G-protein coupled to a membrane receptor is involved in the sequence of events leading to the activation of the Na^+-dependent uridine transporter. The physiological consequences of this regulation remain to be defined.

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