Differential influence of cAMP on the expression of the three subtypes (ATA1, ATA2, and ATA3) of the amino acid transport system A

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Abstract Treatment of HepG2 cells with forskolin led to 60–100% stimulation of system A activity, measured as the Na^+-dependent uptake of α-(methylamino)isobutyric acid. The stimulation was reproducible with cholera toxin and dibutyryl cAMP, and inhibitable by H7, a non-specific protein kinase inhibitor. The stimulatory effect was eliminated by cycloheximide and actinomycin D. The forskolin effect was associated with an increase in the maximal velocity of the transport system, with no change in substrate affinity. These cells express three different subtypes of system A (ATA1, ATA2, and ATA3). Treatment with forskolin increased the steady-state levels of ATA1 and ATA2 mRNAs, but decreased that of ATA3 mRNA.

Key words: Amino acid transport; Subtypes of system a; Transport regulation; Cyclic AMP; Human liver cell line

1. Introduction

System A is an amino acid transport system that mediates Na^+-coupled transport of neutral amino acids [1,2]. The functional activity of this transport system is usually studied using α-(methylamino)isobutyric acid (MeAIB) as the model substrate. Recent cloning studies have revealed that system A actually consists of more than one subtype. Three different transporters have been cloned that have the ability to mediate the Na^+-coupled transport of MeAIB. They are ATA1 [3,4], ATA2 [5–8], and ATA3 [9,10]. ATA1 and ATA2 interact with MeAIB with a K_t value of ~0.5 mM whereas ATA3 exhibits a K_t value for this system A model substrate in the range of 6–10 mM. The three subtypes of system A markedly differ in their tissue expression pattern. ATA1 is expressed primarily in the brain and placenta, ATA2 is expressed in almost every tissue, and ATA3 is expressed almost exclusively in the liver. System A has been shown to be regulated by hormones [11–13]. In the liver, glucagon upregulates the activity of system A through elevation of intracellular levels of cAMP. These regulatory studies have been done prior to our current knowledge that system A actually consists of different subtypes. Therefore, it becomes necessary now to investigate the involvement of the specific subtypes of system A in the stimulatory action of cAMP. Towards this goal, we studied the effect of cAMP on the activity of system A in the human liver cell line HepG2. Even though normal liver expresses predominantly ATA2 and ATA3, the HepG2 liver cell line expresses all three subtypes of system A. In the present study, we analyzed the influence of cAMP on the expression of each of these subtypes of system A in this cell line.

2. Materials and methods

2.1. Materials

The HepG2 human hepatocarcinoma cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). Culture media (minimum essential medium Eagle with Earle's salts and L-glutamine) was obtained from Mediatech, Inc. (Herndon, VA, USA). Penicillin, streptomycin, and trypsin were obtained from Life Technologies, Inc. (Rockville, MD, USA). Fetal bovine serum, forskolin, cholera toxin, dibutyryl cAMP, cycloheximide, actinomycin D and H7 were from Sigma (St. Louis, MO, USA). [^{14}C]MeAIB (specific radioactivity, 50 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). L-[^{3}H]Glutamic acid (specific radioactivity, 24 Ci/mmol), L-[^{3}H]tryptophan (specific radioactivity, 32 Ci/mmol), and L-[^{3}H]arginine (specific radioactivity, 54 Ci/mmol) were purchased from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA).

2.2. Culture of HepG2 cells

The cells were cultured as described in the protocol provided by ATCC. The culture medium (minimum essential medium Eagle with Earle's salts and L-glutamine) was supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and sodium pyruvate (1 mM). Trypsin-released cells were seeded in collagen-coated 24-well plates at a density of 1×10^5 cells/well and allowed to grow as a monolayer. 24 h after subculturing, the medium was replaced with fresh medium that did not contain fetal bovine serum (treatment medium). Treatment with different reagents was carried out for indicated time periods in this treatment medium prior to uptake measurements.

2.3. Uptake measurements in cells

The culture medium was removed by aspiration, and the cells were washed once with the uptake buffer. 0.25 ml of uptake buffer containing radiolabeled substrate (MeAIB, glutamic acid, tryptophan or arginine) was added to the wells and incubated for 10 min at 37°C. Uptake was terminated by aspirating the buffer and subsequently washing the cells twice with fresh uptake buffer. The cells were then lysed with 0.5 ml of 1% SDS in 0.2 N NaOH, and the lysate was transferred to scintillation vials for quantitation of radioactivity. The composition of the uptake buffer was 25 mM HEPES–Tris (pH 8.5), 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl, 0.8 mM $MgSO_4$, 5 mM glucose. Concentration of MeAIB in most uptake experiments was 0.5 mM. Carrier-mediated uptake of MeAIB was calculated by subtracting the uptake measured in the presence of an excess amount of L-alanine (10 mM) from the total uptake measured in the absence of L-alanine.

2.4. Isolation of poly(A)$^+$ RNA and Northern analysis

Total RNA was isolated from control and treated HepG2 cells using Trizol reagent (Life Technologies), and then poly(A)$^+$ RNA

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was isolated from the total RNA by affinity chromatography using oligo(dT) cellulose (Life Technologies). The poly(A)$^+$ RNA samples were size-fractionated on a denaturing formaldehyde-agarose gel and probed with ^{32}P-labeled human ATA1 [4], human ATA2 [8] or human ATA3 [10] cDNA or human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The cDNAs were labeled with [α-^{32}P]dCTP using the ready-to-go oligolabeling kit (Amersham Pharmacia Biotech Inc.). The relative abundance of ATA1, ATA2 or ATA3 mRNA was determined using the following formula: $(a/b)/(c/d)$ where a is the densitometric value (in arbitrary units) of ATA1, ATA2 or ATA3 mRNA band in cells treated with forskolin, b is the densitometric value of the GAPDH mRNA band in cells treated with forskolin, c is the densitometric value of ATA1, ATA2 or ATA3 mRNA band in control cells, and d is the densitometric value of the GAPDH mRNA band in control cells. The relative ATA1, ATA2 or ATA3 mRNA abundance in control cells (c/d) was taken as 1 and the abundance of ATA1, ATA2 or ATA3 mRNA in cells treated with forskolin was expressed in relation to this control value.

3. Results

Forskolin and cholera toxin are the two agents that are widely used to increase intracellular levels of cAMP in mammalian cells. Therefore, we first assessed the influence of forskolin treatment on the transport activity of the amino acid transport system A in HepG2 cells. Cells were treated with 100 μM forskolin for varying periods of time (2–8 h) and then the transport of MeAIB, a specific model substrate for system A, was measured in a Na^+-containing medium. Treatment of the cells with forskolin for 2 h caused a significant stimulation (55 ± 3%) of system A transport activity (Fig. 1A). The stimulatory effect was only slightly increased when the treatment time was extended to 4–8 h. The range of stimulatory effect within this time period was 55–100%. We selected 6 h as the treatment period for subsequent studies. The influence of forskolin on system A activity was dose-dependent, with maximal stimulation observed with 30 μM forskolin (Fig. 1B). The effect of forskolin on system A was specific because the transport of two other amino acids (tryptophan and arginine) was not affected under similar conditions (Fig. 1C). Tryptophan is a preferred substrate for the amino acid transport system L and arginine is a preferred substrate for cationic amino acid transport systems such as y^+, $b^{0,+}$, and y^+L [14–16]. The uptake of glutamate, a preferred substrate for system X^-_{AG}, was however stimulated to a significant extent by forskolin. These data show that forskolin treatment increases the transport activity of system A and X^-_{AG}, but not that of systems L, y^+, $b^{0,+}$, and y^+L.

The stimulatory effect of forskolin on the transport activity of system A was reproducible with cholera toxin and dibutyryl cAMP (Fig. 1D). Treatment of the cells with 100 ng/ml cholera toxin for 6 h increased the transport of MeAIB significantly (30 ± 2%). Under similar conditions, dibutyryl cAMP, a cell-permeable cAMP derivative, also increased the uptake of MeAIB to a significant extent (27 ± 1%). Since the cellular effects of cAMP are mediated principally by protein kinase A, we tested whether the influence of forskolin on system A activity can be blocked by inhibitors of protein kinases. We used H7 for this purpose. H7 is a potent inhibitor of protein kinase A [17]. Treatment of the cells with forskolin (100 μM) in the absence of the protein kinase inhibitor caused an increase of 60 ± 2% in the activity of system A as measured by MeAIB transport. However, when the treatment was carried out in the presence of 50 μM H7, the forskolin effect on the activity of system A was abolished completely (Fig. 1D), indicating that the stimulatory action of forskolin is most likely to be mediated by protein kinase A.

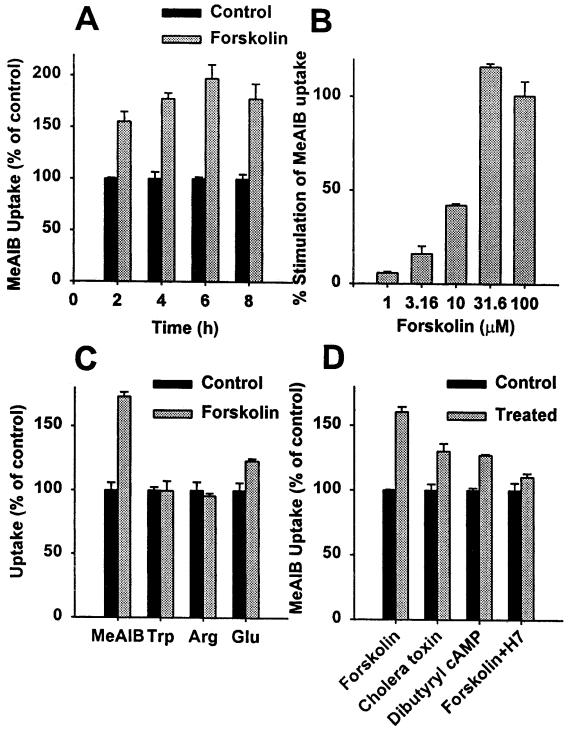

Fig. 1. A: Time course of forskolin-induced effect on MeAIB uptake in HepG2 cells. Cells were treated with or without forskolin (100 μM) for indicated time periods (2–8 h) at 37°C, following which MeAIB uptake was measured at a concentration of 0.5 mM and an incubation period of 10 min. Results are given as percent of corresponding control. B: Dose dependence of the influence of forskolin on MeAIB uptake in HepG2 cells. Cells were treated with increasing concentrations of forskolin for 6 h at 37°C, following which the uptake of MeAIB (0.5 mM) was measured with a 10-min incubation. C: Specificity of forskolin effect in HepG2 cells. Cells were treated with or without forskolin (100 μM) for 6 h at 37°C, following which uptake of MeAIB, L-tryptophan, L-arginine and L-glutamic acid was measured. Final concentration of MeAIB was 0.5 mM and that of L-tryptophan, L-arginine and L-glutamic acid was 20 μM. The incubation period for uptake was 10 min. Results are given as percent of corresponding control. D: Effect of forskolin with or without H7, cholera toxin and dibutyryl cAMP on MeAIB uptake in HepG2 cells. Cells were treated with forskolin (100 μM) in the presence or absence of H7 (50 μM), cholera toxin (100 ng/ml), or dibutyryl cAMP (1 mM) for 6 h at 37°C, following which MeAIB uptake was measured at a concentration of 0.5 mM and an incubation period of 10 min.

To assess the involvement of de novo synthesis of RNA and protein in the effect of forskolin on system A activity, we tested the ability actinomycin D (an inhibitor of transcription) and cycloheximide (an inhibitor of translation) to block the effect of forskolin (Fig. 2A). In the absence of these inhibitors, treatment of the cells with forskolin (100 μM) for 4 h increased MeAIB transport by 60 ± 2%. However, when the treatment was done in the presence of actinomycin D (5 μg/ml) or cycloheximide (50 μg/ml), the stimulatory effect of forskolin was abolished completely. These data show that de novo synthesis of RNA and protein is involved in the forskolin-induced increase in system A activity.

To determine the influence of forskolin on the kinetic parameters of system A, we investigated the saturation kinetics of MeAIB transport in control cells and in forskolin-treated

(forskolin concentration, 100 μM; treatment time, 6 h) cells (Fig. 2B). The transport of MeAIB was saturable in both cases. The values for the Michaelis–Menten constant (K_t) and the maximal velocity (V_{max}) in control cells were 0.44 ± 0.04 mM and 28.2 ± 0.7 nmol/mg protein/10 min, respectively. The corresponding values in forskolin-treated cells were 0.38 ± 0.03 mM and 53.6 ± 1.0 nmol/mg protein/10 min. Therefore, the stimulatory effect of forskolin on system A activity was primarily due to an increase in the maximal velocity of the transport system with no significant change in the substrate affinity.

System A consists of at least three subtypes, known as ATA1, ATA2, and ATA3 [3–10]. To assess the influence of forskolin on the steady-state levels of mRNA specific for each of these three subtypes, we subjected the poly(A)$^+$ RNA samples isolated from control cells and from forskolin-treated (forskolin concentration, 100 μM; treatment time, 6 h) cells to Northern blot analysis (Fig. 3). GAPDH mRNA was used as the internal control. The presence of mRNA for all three subtypes of system A was evident in these cells, suggesting that all three subtypes are expressed in these cells. Treatment with forskolin increased the steady-state mRNA levels for ATA1 and ATA2, but decreased the steady-state mRNA level for ATA3. There were three distinct mRNA species specific for ATA1 (9.0, 2.8, and 0.9 kb). However, forskolin increased the steady-state levels only for 9.0-kb mRNA and 2.8-kb mRNA. There was no change in the levels of 0.9-kb mRNA. The increase in the 9.0-kb mRNA levels was 50% and the increase in the 2.8-kb mRNA levels was 100%. There was only a single mRNA species for ATA2 (4.5 kb) and forskolin increased the levels of this mRNA by 150%. The ATA3-specific mRNA was 4.0 kb in size and forskolin decreased the levels of this mRNA by 50%. These data show that forskolin has differential effects on the expression of the three subtypes of system A.

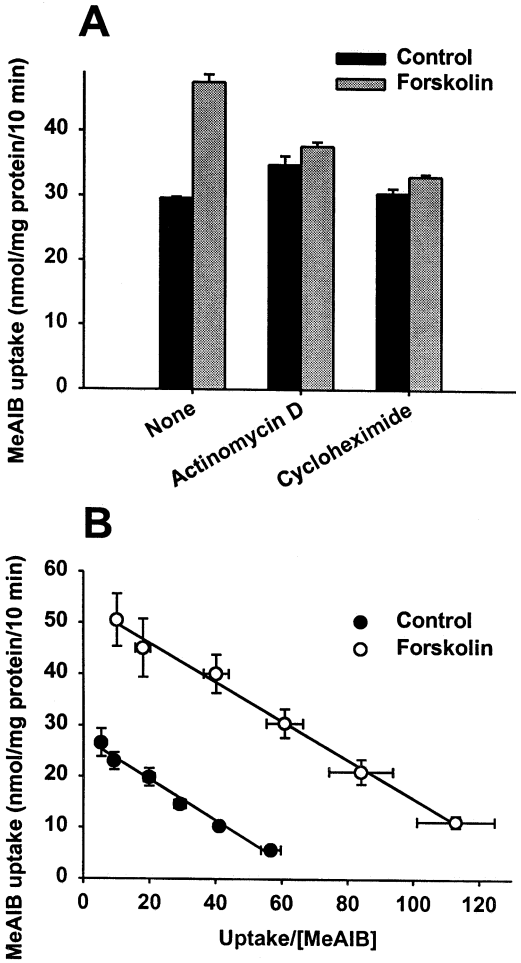

Fig. 2. A: Effect of actinomycin D and cycloheximide on forskolin-induced stimulation of MeAIB uptake in HepG2 cells. Cells were treated with or without forskolin (100 μM) in the presence of actinomycin D (5 μg/ml) or cycloheximide (50 μg/ml) for 4 h at 37°C. Following the treatment, MeAIB uptake was measured at a concentration of 0.5 mM and an incubation period of 10 min. B: Effect of forskolin on the saturation kinetics of MeAIB uptake in HepG2 cells. Cells were treated with or without forskolin (100 μM) for 6 h at 37°C, following which MeAIB uptake was measured over the MeAIB concentration range of 0.1–10 mM and with an incubation period of 10 min. Results represent Eadie–Hofstee transformation of the uptake data.

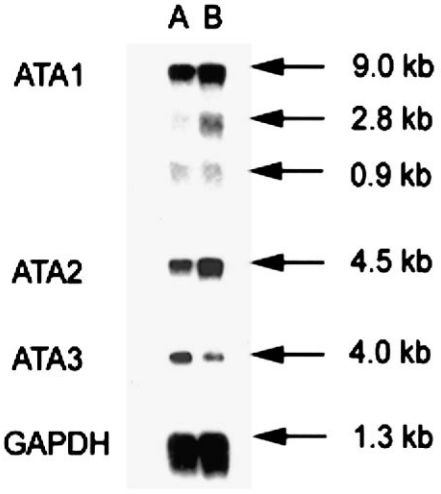

Fig. 3. Effect of forskolin on steady-state levels of ATA1, ATA2, ATA3 and GAPDH mRNA in HepG2 cells. Cells were treated with (lane B) or without (lane A) forskolin (100 μM) for 6 h at 37°C. Poly(A)$^+$ RNA was isolated from control and treated cells. The size-fractionated mRNA was probed with ^{32}P-labeled human ATA1, human ATA2, human ATA3 and human GAPDH cDNA by sequential hybridization.

4. Discussion

This represents the first report on the influence of cAMP on the expression of the amino acid transport system A at the molecular level. Previous studies have clearly shown that the transport activity of system A in hepatocytes is enhanced by cAMP and that the process involves de novo synthesis of RNA and protein [11–13]. When these studies were done, it was not known that system A actually consists of different subtypes. Recent data indicate that liver expresses at least two of the three known subtypes of system A [5,8–10]. Northern blot analysis has provided evidence for the presence of ATA2 mRNA and ATA3 mRNA in the liver [5,8–10]. This is supported by our recent isolation of cDNAs for ATA2 and ATA3 from a HepG2 cDNA library [8,10]. The mRNA for ATA1 was not detectable in the liver by Northern hybridization [3]. However, the present studies show that HepG2 cells express all three subtypes of system A.

Since the identification of subtypes within system A, several reports have appeared in the literature on the regulation of this transport system at the molecular level [18–21]. However, all these studies have focused on one particular subtype, namely ATA2. The expression of ATA2 is induced by amino acid deprivation in human fibroblasts[18], rat C6 glioma cells [19], and rat skeletal muscle cells [20] and also by hypertonicity in human endothelial cells [21]. In all cases, the induction is associated with a parallel increase in the steady-state levels of ATA2 mRNA. The present report describes for the first time the changes in the expression of all three subtypes of system A in response to a specific regulatory signal. The activity of system A in HepG2 cells is enhanced by agents that increase intracellular levels of cAMP as well as by a cell-permeable cAMP derivative. Since HepG2 cells express all three subtypes of system A, it becomes necessary to establish the identity of the subtype that is subject to regulation by cAMP. The present studies show that cAMP induces the expression of ATA1 as well as the expression of ATA2. In contrast, the expression of ATA3 is suppressed by cAMP. Based on the known kinetic characteristics of the three subtypes of system A [4,8,10], we believe that the transport of MeAIB observed in HepG2 cells is mediated predominantly by ATA1 and ATA2. The contribution of ATA3 to the observed transport is likely to be minimal. This is because the affinity of ATA3 for MeAIB is at least one order of magnitude lower that the affinity of ATA1 or ATA2 for the same substrate [4,8,10]. Kinetic analysis of MeAIB transport in these cells suggests the apparent involvement of a single saturable transport process. This is because ATA1 and ATA2 exhibit similar saturation kinetics. The Michaelis–Menten constant for human ATA1 is 0.89 ± 0.12 mM [4], a value similar to the Michaelis–Menten constant for human ATA2 (0.39 ± 0.05 mM) [8]. These two values are so close to each other that they cannot be differentiated by analysis of saturation kinetics. Therefore, we conclude that the increase in MeAIB transport induced by cAMP in HepG2 cells is due to an increase in the expression of ATA1 as well as ATA2.

The present studies show that cAMP affects differentially the expression of the three subtypes of system A. This second messenger enhances the expression of ATA1 and ATA2 but suppresses the expression of ATA3. ATA1 is expressed primarily in the brain, placenta, and heart whereas ATA2 is expressed ubiquitously. Whether or not cAMP will have a similar stimulatory effect on the expression of ATA1 and ATA2 in all tissues expressing the two subtypes remains to be seen.

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