Regulation of the efflux of putrescine and cadaverine from rapidly growing cultured RAW 264 cells by extracellular putrescine

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Cultures of the macrophage-like RAW 264 cells were adapted to divide normally in a synthetic serum-supplemented culture medium lacking any polyamines and diamine oxidase activity. These rapidly dividing cells actively effluxed large amounts of putrescine and cadaverine, compared with the intracellular levels, into the culture medium. The efflux of putrescine was stimulated by the amino acid ornithine, whereas efflux of cadaverine was inhibited. Relatively low levels of spermidine and *N*¹-acetyl-spermidine, compared with those of exported putrescine, were observed to accumulate in the culture medium. A careful analysis of the changes in the intracellular concentration of putrescine relative to the steady-state net rate of putrescine export, as the doubling time of the cultures increased from 16 h to 22 h, indicated that an inverse relationship existed between these two

parameters. As the intracellular putrescine concentrations increased, the net rate of putrescine export decreased markedly. Determination of the rate of putrescine uptake indicated that putrescine uptake also decreased significantly as the cultures neared confluency, and at no time during the growth of the culture did the rate of putrescine uptake approximate to the high rate of putrescine efflux. The decrease in the putrescine export rate seen as the cells grew toward confluency was determined to be primarily due to the inhibitory effect of the effluxed putrescine in the medium ($K_i = 2 \mu\text{M}$), and not to contact inhibition. The data suggested that the efflux of putrescine and cadaverine is not mediated to a significant degree by a process involving simple diffusion.

INTRODUCTION

The polyamines are naturally occurring aliphatic amines having a positive charge on each nitrogen atom at physiological pH [1]. Putrescine is synthesized from the basic amino acid ornithine via a decarboxylation reaction catalysed by the highly regulated enzyme ornithine decarboxylase (ODC; EC 4.1.1.17) [1]. Spermidine and spermine are synthesized from putrescine through the sequential addition of a propylamine moiety from *S*-adenosylmethionine [1,2]. Polyamines are believed to function intracellularly by virtue of their high positive charge-to-mass ratio, which enables them to bind tightly to cellular anionic macromolecules such as DNA, RNA or membrane phospholipids [3]. Although the precise physiological functions of these amines have not been defined, they have been proved to be essential for growth, proliferation and differentiation of virtually all eukaryotic and prokaryotic cells. As such, increases in polyamine biosynthesis usually accompany increases in cell growth in response to hormones and growth factors [4].

Factors which influence the regulation of intracellular polyamine levels include biosynthesis, turnover, uptake into the cell, and efflux of these amines from the cell [3]. Polyamine biosynthesis has been known to be a highly regulated process at many steps, including gene transcription, mRNA turnover, and translation, and post-translational regulation [5]. The turnover of the polyamines is regulated through a series of acetylation/oxidation reactions providing the polyamine with a relatively long half-life under most circumstances in comparison with other biological amines such as the catecholamines. Polyamine uptake has been characterized in a variety of systems, including a number of mammalian cells [6] and *Neurospora* [7], and the proteins involved in the uptake system were identified and cloned in prokaryotes such as *Escherichia coli* [8,9]. Current data suggest the presence of at least two different uptake systems capable of

transporting putrescine and spermidine into mammalian cells. Polyamine efflux or export has also been investigated in a number of laboratories, although, due to the more difficult nature of studying such phenomena, it has not been as clearly defined as the uptake mechanism.

A variety of cultured mammalian cells, such as human colon tumour HT 29 cells [10], Chinese-hamster ovary cells [11,12] and human melanoma MALME-3 cells [13], were found to export polyamines (the majority in the form of spermidine and *N*¹-acetylspermidine). Cultures of baby-hamster kidney cells (BHK-21/C13 cells) were also found to efflux polyamines primarily in the form of spermidine into the extracellular medium [14,15].

We have observed previously that the cultured macrophage-like RAW 264 cells constitutively exported relatively large amounts of putrescine and cadaverine into the culture medium [16]. Export of putrescine and cadaverine was stimulated by bacterial lipopolysaccharide, interferon- γ and/or ornithine, and was markedly inhibited by α -difluoromethylornithine, an irreversible ornithine decarboxylase inhibitor [16,17]. We have further characterized the export of putrescine and cadaverine in this and a number of other cell lines [18], and have observed that such export is not dependent on intracellular or extracellular Ca^{2+} , nor is it affected by changes in intracellular pH [19]. In addition, the drugs verapamil and quinine inhibited markedly the export of putrescine and cadaverine from a number of cultured cell lines, independently of any effect upon the intracellular pools of the polyamines [19]. This inhibition appears to be due to the direct binding of these drugs to an integral membrane protein and not to the ability of these agents to alter Ca^{2+} influx.

It has been suggested that, during periods of rapid cell growth, the uptake of polyamines is stimulated, whereas the efflux or export of polyamines from the cell is inhibited [14,15]. This has been postulated as a mechanism whereby cells maximize the

Abbreviation used: ODC, ornithine decarboxylase.

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intracellular accumulation of polyamines during certain stages of the cell cycle (late G₁) when such amines are required before S-phase. Most of our previous studies described above [16–18] were performed in quiescent, non-growing, cultures of mammalian cells grown in serum-free medium. Putrescine/cadaverine efflux was investigated in this manner in order to minimize the degradation of extracellular diamines by the enzyme diamine oxidase, which is present in virtually all preparations of fetal-calf serum [20]. It has been suggested further that the process involved in putrescine efflux from the cell is one of simple diffusion through the cell membrane and does not involve an integral membrane export protein [7,21]. In order to determine accurately the rate of putrescine and cadaverine export in cultured cells during the exponential phase of cell growth, the RAW 264 cells were adapted for growth in a synthetic serum supplement which lacked detectable activity of diamine oxidase. In this manner, the measured steady-state rates of putrescine and cadaverine export in cells actively traversing the cell cycle and growing rapidly during exponential phase and stationary phase could be compared with the intracellular accumulation of the polyamines. If simple diffusion of putrescine through the membrane were the primary mechanism mediating efflux from the cell, the rate of putrescine export would be proportional to the intracellular putrescine concentration.

MATERIALS AND METHODS

RAW 264 cell culture

The mouse monocyte-macrophage RAW 264 cell line was obtained from American Type Culture Collection (Rockville, MD, U.S.A.). Cells were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD, U.S.A.) supplemented with 10% (v/v) fetal-bovine serum (Gibco BRL). Cells were grown in 100 mm-diameter plastic Petri dishes at 37 °C in humidified air containing 5% CO₂. To rule out the presence of any contaminating mycoplasma, the cells were screened with the Mycoplasma Detection Test (Gen-Probe, San Diego, CA, U.S.A.) as described by the manufacturer, and showed a negative result for mycoplasma. The cells were frozen in liquid N₂ and grown in culture for no longer than 2 weeks before each experiment.

Serum has been reported to contain diamine oxidase [20]. Such enzymic activity markedly decreases the level of putrescine that can be measured in the culture medium [20]. Under some circumstances cultured cells can be grown in aminoguanidine (1 mM) in order to inhibit diamine oxidase effectively. However, this compound was not employed in our studies, because it can be toxic to cells and might alter polyamine uptake, export or intracellular metabolism. In addition, we have found significant amounts of the diamines putrescine and cadaverine (up to 10 µM) in different batches of commercial fetal-bovine serum, which even extensive dialysis failed to remove. For these reasons RAW 264 cells were gradually adapted over a 3-week period to grow in culture medium containing only 2% fetal-bovine serum and 2% TCM, a defined serum replacement (Celox, Hopkins, MN, U.S.A.), which was supplemented with 2 mM glutamine and 10 mM Hepes. All experiments described here were done in the absence or presence of 1.0 mM exogenous ornithine (Fluka, Ronkonkoma, NY, U.S.A.) or up to 2 µM exogenous putrescine (Sigma Chemical Co., St Louis, MO, U.S.A.). Exogenous putrescine added to this culture medium remained stable for 7 days when incubated at 37 °C and was not degraded by the residual diamine oxidase activity present in the 2% fetal-bovine serum.

Sample collection

Cells were rapidly washed with PBS, removed from the plates by scraping, and pooled into ice-cooled conical plastic tubes. The tubes were then spun in a clinical centrifuge and the supernatant was decanted. To the cells, 200 µl of 0.5 M HClO₄ was added. The cells were homogenized by sonication and, after further centrifugation at 10000 g for 10 min, the supernatant was further subjected to analyses for intracellular polyamine and amino acid as described below. The precipitate was dissolved in 0.5 M NaOH for protein determination by the Bradford [22] method, with bovine serum albumin as a standard. The cell numbers were also determined with a haemocytometer. All polyamine values were normalized to the amounts of acid-precipitable protein or the cell numbers determined in this manner.

Determination of intracellular and extracellular polyamine levels

The intracellular polyamine analysis was performed by a h.p.l.c. procedure employing pre-column derivative-formation with dansyl chloride as described in [23]; 250 µl of acid-soluble supernatants was neutralized and treated with 200 µl of 5 mg/ml dansyl chloride (Fluka) in acetone for 15 min at 70 °C. The reacted samples were then pre-chromatographed on small C₁₈ hydrophobic columns (Analytichem International, Harbor City, CA, U.S.A.), eluted with 1.5 ml of methanol and subsequently analysed by h.p.l.c.

Analysis of extracellular polyamines in culture medium was performed by using a clean-up procedure before pre-column derivative formation with dansyl chloride as described in [24]. Briefly, the culture medium sample was subjected to chromatography on Biorex-70 (Bio-Rad, Richmond, CA, U.S.A.), a cation-exchange resin, packed to a 0.7 ml resin bed volume in a 12 ml plastic syringe. The column was washed once with 2.5 ml of 0.145 M NH₄OH, and the extracellular polyamines were eluted with 3 ml of 4.0 M NH₄OH. The eluted samples were rotary-evaporated to dryness, neutralized, and treated with dansyl chloride to a final concentration of 2.5 mg/ml. The modified sample was then subjected to h.p.l.c. analysis. By this procedure, putrescine, spermidine, spermine and all conjugated derivatives of polyamines, i.e. acetylputrescine, N¹- and N⁸-acetylspermidine and acetylspermine, could be quantified in 10 ml of culture medium with a limit of detectability as low as 0.1 nmol/mg total cellular protein.

Determination of putrescine uptake

RAW 264 cells were seeded in 60 15 mm-diam. dishes at ~ 7 × 10⁴ cells/dish in the absence or in the presence of exogenous ornithine (1.0 mM). The growth rate (doubling time) and the cell density of RAW 264 cells under these dishes was identical with that grown in 100 mm-diameter culture dishes. At the indicated time, cells were labelled for 30 min with [³H]putrescine (2 µCi/ml; sp. radioactivity 12.6 Ci/mmol) and sufficient unlabelled putrescine to give a final concentration of 1 µM. (This concentration was chosen since it corresponded to the observed amount of putrescine in the culture medium supplemented with ornithine at day 1 of culture). The cells were then washed with 3 × 1 ml of cold PBS containing 100 µM putrescine to minimize non-specific binding of the labelled putrescine, and harvested. A fraction (800 µl) of the supernatant was analysed for ³H with a scintillation counter, and the remaining fraction was subjected to protein analysis. Under these conditions, uptake was linear with time up to 2 h and was also linear from 0 to 2 µM total putrescine (results not shown).

Data analysis

All data were represented in terms of amount of polyamine or amino acid per cell number of per mg of total protein (which was directly proportional to the cell number). Calculations of the rate of putrescine or cadaverine export (in terms of nmol/cell number per day) were carried out by dividing the measured amount of diamine effluxed during the previous 24 h period by the calculated number of cells present 12 h before the indicated day in culture. The determination of approximate polyamine concentrations inside the cell was performed by assuming a cell diameter of 12 μm and a calculated cell volume of 905 μm^3 .

RESULTS

Growth of RAW 264 cells in culture medium containing fetal-bovine serum or a synthetic serum supplement

As stated previously, fetal-bovine serum contains diamine oxidases that can decrease the level of measurable putrescine or cadaverine [20]. In addition, serum has been found to contain measurable levels of the polyamines (primarily in the form of putrescine and cadaverine). We observed up to 10 μM concentrations of these amines in various lots of fetal-calf serum (results not shown). For this reason, the monocytic-leukaemic RAW 264 cells were gradually switched from culture medium containing 10% fetal-bovine serum to that containing 2% fetal bovine serum and TCM, a synthetic, defined, serum replacement, during a 3-week period (see the Materials and methods section). The RAW 264 cells grown under these conditions had a growth rate that was virtually indistinguishable from those cells grown in culture medium supplemented with 10% fetal-bovine serum (Figure 1). Since the presence of ornithine in culture medium had been previously reported to lead to markedly increased rates of putrescine export [16,18], the growth of the RAW 264 cells in the synthetic serum supplement was also measured in the presence of 1 mM ornithine. The presence of ornithine did not appear to alter the growth of these cells, as shown in Figure 1. Between days 0 and 2–3, the cultures of RAW 264 cells were in exponential-

Figure 1 Changes in the cell numbers of rapidly growing cultures of RAW 264 cells in the absence or presence of exogenous ornithine

RAW 264 cells were seeded at low density ($\sim 4 \times 10^5$ cells/dish) in culture medium containing 2% fetal-bovine serum in addition to 2% TCM defined serum replacement (Celox) in the absence or presence of exogenous ornithine. Cell numbers were determined with a haemocytometer daily throughout a 4-day period. Before the experiment, the cells were gradually adapted from 10% fetal-bovine serum to 2% serum and serum replacement over a 3-week period. Each symbol represents the mean $\pm$ S.E.M. of three determinations from three culture dishes. For many data points the size of the SEM was less than the size of the symbol as illustrated on the Figure.

phase growth and began to slow their growth rate and enter 'stationary phase' between days 3 and 4. The 'growth' of the cell culture could be observed to decrease as the doubling time under all conditions was increased from 12 h between days 0 and 2, to 18 h between days 2 and 3, to 22 h between days 3 and 4. RAW 264 cells grown in up to 20% fetal-bovine serum exhibited a similar growth curve to those shown in Figure 1 (results not shown).

Alterations in intracellular and extracellular polyamines during growth of RAW 264 cells

The amounts of intracellular and extracellular polyamines were determined in the cultures of RAW 264 cells on different days in culture corresponding to the increasing cell numbers shown in the growth curve (Figure 1). In order to discuss more easily the amount of intracellular putrescine relative to export, the intracellular amounts of the polyamines were represented both as the total amount present in the culture dish and as the amount present on a per-cell basis (i.e. per mg of total cellular protein). The total intracellular putrescine per dish was relatively low during days 0–2 in culture, corresponding to exponential-phase growth (see Figures 1 and 2). Between days 2 and 4 in culture, the putrescine levels increased significantly, reaching approx. 1.5 nmol/dish. During this period, the intracellular levels of putrescine were increased several-fold in the cultures receiving ornithine, in comparison with the control cells. The total amount of both intracellular spermidine and spermine increased significantly from day 0 to day 4 in culture, as shown in Figure 2(b). The cells grown in the presence of ornithine showed a small difference, compared with control cultures, in their levels of spermidine and spermine. The total amount of spermidine and spermine in culture was significantly greater (~ 25 -fold) than the amount of intracellular putrescine.

When the levels of the polyamines were represented as nmol/mg of total cellular protein (Figures 2c and 2d), the amounts of spermidine and spermine remained relatively constant, in comparison to putrescine, throughout the 4-day period of cell growth. However, the amount of putrescine per cell remained relatively low throughout days 0–2 in culture, and increased 10–20-fold when the cells entered stationary phase between days 3 and 4.

When the culture medium was analysed for extracellular polyamines, significant levels of putrescine and cadaverine were observed throughout the culture period (Figures 3 and 4), whereas considerably smaller amounts of spermidine and *N*¹-acetyl-spermidine were also found (Figures 5a and 5b). In the control cultures, the level of putrescine accumulated in an approximately linear manner up to a value of 7.5 nmol of total putrescine per dish. In cells cultured in the presence of ornithine, the total amount of effluxed putrescine was increased by approx. 3-fold, to 22 nmol/dish. In a similar manner, the control cultures also exhibited an efflux of cadaverine into the culture media, which reached 5 nmol/dish after 4 days of culture. As seen previously [16], in the presence of ornithine, the RAW 264 cells did not export any cadaverine (we have reported previously [17,19] that cadaverine levels are below the limit of detectability inside the cell).

Analysis of the net steady-state rates of putrescine and cadaverine export throughout a 4-day period of RAW 264 cell growth

The net rates of putrescine and cadaverine export were calculated from the data shown in Figures 3(a) and 4(a) and represented as the amount of putrescine or cadaverine exported per cell per

Figure 2 Alterations in the levels of intracellular putrescine (a, c) and intracellular spermidine (SD) and spermine (SP) (b, d) during growth of RAW 264 cells

RAW 264 cells were seeded at $\sim 4 \times 10^5$ cells/dish in medium containing 2% serum plus 2% TCM serum supplement, in the absence (white symbols) or in the presence (black symbols) of exogenous ornithine (Orn; 1.0 mM). At the indicated time, the cells were harvested, and the concentrations of polyamines were determined as described in the Materials and methods section. In (a) and (b) the data are represented as the total amount of intracellular polyamines present in the culture dish, whereas in (c) and (d) the data are represented as the amount of intracellular polyamine present per mg of total cellular protein. Total cellular protein was directly proportional to cell number throughout the 4 days of culture (results not shown). Each point represents the mean $\pm$ S.E.M. of three determinations from three culture dishes. For many data points the size of the S.E.M. was less than the size of the symbol as illustrated on the Figure.

24h period (Figures 3b and 4b). When the data are represented in this manner, it is apparent that the steady-state rate of putrescine and cadaverine export was the highest in exponential phase, or days 0–2, of culture when the cells were the most rapidly proliferating. The rate of diamine export decreased significantly as the cells reached near-confluency at day 4. Ornithine resulted in a marked increase in the putrescine export rate in comparison with the control culture throughout the 4-day culture period. The decrease in the rate of export of putrescine observed as the days in culture increased was particularly evident in those cultures which were cultured with ornithine. The rate of export decreased 5-fold as the cells were cultured from day 1 to day 4 and reached stationary phase. A similar decrease in cadaverine export rates was also observed during this period. The addition of ornithine to the culture medium markedly decreased the rate of cadaverine export, presumably by competing with lysine for ODC [17].

As stated in the Introduction, other investigators have proposed that the export, efflux or loss of putrescine and polyamines from the cell involves a process of simple diffusion of these amines through the membrane [7]. If this were the case, one would predict that the rate of putrescine export would be proportional to the intracellular concentration of this diamine, since simple diffusion through the membrane is primarily driven or regulated by the concentration gradient which exists across the membrane [25]. In order to ascertain the relationship between the intracellular concentration of putrescine and the corre-

sponding export rate, the total intracellular putrescine concentration was calculated (as described in the legend to Figure 6 and in the Materials and methods section) at various times during culture of the RAW 264 cells and compared with the observed rate of putrescine export shown in Figure 3.

When the data are represented in this manner, it is clear that the total intracellular putrescine concentration was not directly proportional to the observed net steady-state rate of putrescine export. In contrast, those cells possessing the lowest concentration of intracellular putrescine (cells during days 1 and 2 of growth) had markedly higher rates of putrescine export, and the lowest level of intracellular putrescine, than, for example, the cells during days 3 and 4, which had significantly higher intracellular putrescine and lower rates of export. Cells on day 4 had approx. 25 times greater concentration of intracellular putrescine than those cultures on day 1, yet rates of putrescine export were 3–5-fold less. Such observations argue against the significant involvement of simple diffusion of putrescine through the membrane as a mechanism to account for the efflux observed (see the Discussion section).

Determination of putrescine uptake rates and comparison with the rates of putrescine export

The data shown in Figure 6 represent the steady-state net rate of putrescine export and do not take into consideration the con-

Figure 3 Alterations in the levels of exported putrescine and the steady-state rate of putrescine export throughout a 4-day growth period of RAW 264 cells

RAW 264 cells were seeded at $\sim 4 \times 10^5$ cells/dish in medium containing 2% serum plus 2% TCM serum supplement, in the absence or presence of exogenous ornithine (Orn; 1.0 mM). At the indicated time, cells were harvested, and the levels of putrescine effluxed were determined as described in the Materials and methods section. The rate of putrescine export was determined by dividing the measured amount of putrescine exported during the previous 24 h period by the calculated number of cells present 12 h before the indicated day in culture. [For example, the rate of putrescine export indicated at day 2 of culture in (b) was determined from the amount of putrescine export between days 1–2 (the difference) and the numbers of cells present at $1\frac{1}{2}$ days.] Each point represents the mean $\pm$ S.E.M. of three determinations from three culture dishes. For many data points the size of the S.E.M. was less than the size of the symbol as illustrated on the Figure.

tribution of putrescine uptake relative to the export of putrescine. For this reason, the uptake rate for putrescine was determined throughout days 0–4 in culture by using a 30 min incubation with [3 H]putrescine at a total concentration of 1 μ M as described in the Materials and methods section and in the legend to Figure 7. The rate of putrescine uptake on a per-cell basis decreased markedly between days 1 and 4 (Figure 7) of culture in a similar manner to the net rate of export (Figure 6). The actual rate of putrescine export at the various days of culture was calculated from the putrescine uptake rate measured in the presence of 1 μ M putrescine, adjusted to take into account the actual putrescine concentration in the culture medium throughout days 1–4 (shown in Figure 3a). In this regard, the cells were exporting markedly more putrescine (up to 10 times) than was taken up throughout days 1–4 in culture in the presence and absence of

Figure 4 Alterations in the levels of exported cadaverine and the steady-state rate of putrescine export throughout a 4-day period of RAW 264 cell growth

RAW 264 cells were seeded at $\sim 4 \times 10^5$ cells/dish in medium containing 2% serum plus 2% TCM serum supplement, in the absence or presence of exogenous ornithine (Orn; 1.0 mM). At the indicated time, cells were harvested, and the levels of cadaverine effluxed were determined as described in the Materials and methods section. The rate of cadaverine export was determined by dividing the measured amount of cadaverine export occurring during the preceding day by the cell number calculated to be $\frac{1}{2}$ day before. Each point represents the mean $\pm$ S.E.M. of three determinations from three culture dishes. For many data points the size of the S.E.M. was less than the size of the symbol illustrated on the Figure.

ornithine (Table 1). When the true rate of putrescine export is estimated by subtracting the putrescine uptake rate from the net rate of putrescine export, the conclusions made above arguing against a simple diffusion mechanism for putrescine efflux remain valid. The RAW 264 cells, especially early in culture, still exhibited a higher true rate of putrescine export when the intracellular putrescine concentrations were the lowest.

Potential effect of cell–cell contact and extracellular putrescine on the efflux of putrescine in the RAW 264 cells

There are at least two potential mechanisms which could explain why the rapidly growing sparse cultures of RAW 264 cells exhibited a markedly higher rate of putrescine export as compared with the slower-growing, more confluent, cells. It was conceivable that the rate of putrescine export was regulated by cell-to-cell contact. The RAW 264 cells were seeded as very sparse cultures on day 0. They were plated as single cells well separated from

Figure 5 Changes in the levels of exported spermidine (a) and N^1 -acetylspermidine (b) by dividing RAW 264 cells

RAW 264 cells were seeded at $\sim 4 \times 10^5$ cells/dish in medium containing 2% serum plus 2% TCM serum supplement, in the absence (○) or in the presence (●) of exogenous ornithine (Orn; 1.0 mM). At the indicated time, the cells were harvested, and the levels of extracellular spermidine (a) and N^1 -acetylspermidine (b) were determined as described in the Materials and methods section. Each point represents the mean $\pm$ S.E.M. of three determinations from three culture dishes. For many data points the size of the S.E.M. was less than the size of the symbol as illustrated on the Figure.

each other across the culture dish. After each successive day in culture of these monolayer cells, the degree of contact of one cell to another was greater as the cells doubled every 12–22 h. Thus the percentage of cells in contact with other cells increased with days in culture as the size of the colonies originally grown from single cells increased. It was conceivable that the contact of one cell to another led to a decrease in putrescine export in a manner similar to what has been shown for a number of other cellular processes (i.e., contact inhibition) [26,27].

In order to test this hypothesis, the RAW 264 cells were seeded initially into culture dishes at three different cell numbers (cell densities) equivalent to the cell numbers observed in days 1, 2 and 3 shown in Figure 1. The rate of putrescine export was determined for a 25 h period following the seeding of the cells. In this manner each culture should have the equivalent number of cells as shown in Figure 3(b) at days 1, 2 and 3 in culture. However, these cells, in comparison, would only be in minimal contact with other cells during the initial 24 h period in which putrescine export was measured. If putrescine export were

Figure 6 Comparison of the rate of putrescine export and the intracellular concentrations of putrescine during the growth of RAW 264 cells

See the legends to Figures 2 and 3 for a detailed explanation of the measurement of the putrescine export rate. Approximate putrescine concentrations inside the cells were calculated by dividing the measured amount of intracellular putrescine (in nmol) by the calculated cell volume of $905 \mu\text{m}^3$ (assuming a cell diameter of $12 \mu\text{m}$). The days in culture (and the corresponding cell numbers) are also indicated for each data point.

Figure 7 Uptake of putrescine by dividing RAW 264 cells

RAW 264 cells were seeded in the absence (○) or in the presence (●) of exogenous ornithine (Orn; 1.0 mM) as described in the Materials and methods section. At the indicated time, cells were rinsed and labelled with [^3H]putrescine (total concn. $1 \mu\text{M}$) for 30 min, harvested, and counted for total labelled putrescine taken up during this period. The data was then represented as total putrescine taken up (in nmol/cell per day), to facilitate comparison with the net export rates shown in Figure 6. Each point represents the mean $\pm$ S.E.M. of three determinations from three cultures dishes. For many data points the size of the S.E.M. was less than the size of the symbol as illustrated on the Figure.

contact-inhibited, one would expect to observe no change in the high rate of putrescine export in the three different culture densities in this experiment. However, as shown in Figure 8, in both the cultures incubated with ornithine and in the control cultures the rate of putrescine export is significantly decreased during the 24 h period after the plating of increasing amounts of numbers of cells into the culture dish. This observation was not consistent with contact inhibition of putrescine export.

Table 1 Comparison of rates of putrescine uptake and export during growth of RAW 264 cells

Abbreviations: Put, putrescine; Orn, ornithine.

Period of culture (days)	Culture-medium Put concn. (μM)*		$10^7 \times$ Rate of Put uptake (nmol/cell per day)		Calculated from media Put concn.†		$10^7 \times$ Net rate of Put export§ (nmol/cell per day)	
	—Orn	+Orn	—Orn	+Orn	—Orn	+Orn	—Orn	+Orn
1	0.22 ± 0.01	1.02 ± 0.03	24.22 ± 0.26	28.69 ± 0.60	5.40	29.26	39.83 ± 0.01	185.29 ± 0.41
2	0.40 ± 0.00	1.53 ± 0.04	20.68 ± 0.73	13.03 ± 1.87	8.27	19.94	26.64 ± 0.07	101.61 ± 0.23
3	0.50 ± 0.05	1.85 ± 0.06	2.34 ± 0.04	3.37 ± 1.17	1.17	6.23	16.23 ± 1.45	60.35 ± 2.10
4	0.76 ± 0.01	2.22 ± 0.01	0.96 ± 0.01	0.63 ± 0.01	0.72	1.39	15.82 ± 0.21	39.86 ± 5.75

* Data obtained from Figure 3(a).

† Data obtained from Figure 7. See legend to Figure 7 and the Material and methods for experimental details.

‡ Since putrescine uptake was linear between 0 and $2 \mu\text{M}$ (results not shown), using the concentration of media (exported) putrescine, we calculated the real or actual rate of putrescine uptaken by the RAW 264 cells during growth.

§ Data obtained from Figure 6.

Figure 8 Changes in the rate of putrescine efflux in RAW 264 cells seeded at different densities

RAW 264 cells were grown in medium containing 2% serum plus 2% TCM defined serum supplement, in the absence (○) or presence (●) of exogenous ornithine (Orn; 1.0 mM) at the indicated cell densities. After a 24 h period, the cells were harvested, and the level of putrescine in the culture medium was determined as described in the Materials and methods section. The rate of putrescine exported was calculated by dividing the amount of exported putrescine by the number of cells present 12 h before. Each point represents the mean $\pm$ S.E.M. of three determinations from three cultures dishes. For many data points the size of the S.E.M. was less than the size of the symbol as illustrated on the Figure.

The level of putrescine in the culture medium was increasing as cell numbers increased, upon each successive day in culture, due to the export or efflux of this diamine from the cells. It was conceivable also that the effluxed exogenous putrescine was inhibiting the export of putrescine from the RAW 264 cells. In order to determine whether extracellular putrescine was inhibiting putrescine efflux, 3×10^6 RAW 264 cells were seeded at time 0, and the effect of increasing concentrations of putrescine on the efflux of putrescine from the cells was determined during a 24 h period. As shown in Figure 9, putrescine in culture media was able to give a dose-dependent decrease in putrescine export, with a K_i of approx. $2 \mu\text{M}$. Thus it would appear that the marked decrease in the rate of putrescine export seen as cells reached confluency was due, for the most part, to the inhibition of the

Figure 9 Alterations in the amount of putrescine effluxed by RAW 264 cells in the presence of different concentrations of exogenous putrescine

RAW 264 cells were seeded in medium containing 2% serum, plus 2% TCM serum supplement and exogenous ornithine (1.0 mM), at a density corresponding to that of a 3-day culture (i.e. 3×10^6 cells). Different concentrations of exogenous putrescine were then added to the culture medium. The cells were harvested after a 24 h period, and the levels of putrescine were determined as described in the Materials and methods section. Changes in the amount of putrescine effluxed (in the presence of exogenous putrescine) were calculated by subtracting the amounts of exogenous putrescine added from the total putrescine levels found in the culture medium after the 24 h period. Each point represents the mean $\pm$ S.E.M. of three determinations from three cultures dishes.

export process brought about by extracellular putrescine exported into the media by the same cells.

DISCUSSION

The data presented here suggest that dividing mammalian cells actively effluxed the diamines putrescine and cadaverine into the extracellular space. The export of putrescine was enhanced by ornithine, whereas that of cadaverine was not (Figures 3 and 4). Only small amounts of spermidine and N^1 -acetylspermidine, relative to putrescine and cadaverine, were observed to accumulate in the culture medium (see below). Although the

absolute amounts of extracellular putrescine and cadaverine in the medium increased as the cell cultures grew toward confluency, the steady-state rates of export of the diamine per cell per day decreased significantly (Figures 3 and 4). These data were inconsistent with previous observations in which growing cells were observed to efflux only small amounts of the polyamines [14]. Specifically, these investigators found that confluent, non-growing, cultures of BHK-21/C13 cells continuously excreted polyamines, but when the cell growth was reinitiated by addition of fresh serum, the cultures immediately increased polyamine uptake and considerably decreased polyamine efflux [14]. It is conceivable that the decrease in polyamine efflux observed by these investigators was mediated by the presence of high levels of putrescine found in commercially available serum. We have found previously that different lots of fetal-bovine serum contain variable amounts of putrescine and cadaverine, up to 10 μM in concentration. The presence of even μM levels of putrescine in serum could result in significant under-estimation of the measurement of polyamine export in cell cultures, due to the marked inhibitory effect of extracellular putrescine on putrescine export (Figure 9; see below). The results of this and previous studies [16,18] suggest strongly that putrescine and cadaverine effluxes occur in both quiescent and dividing cultures of mammalian cells at similar rates.

At no time did we find any significant accumulation of either spermidine or N^1 -acetylspermidine in the culture medium of growing cultures of RAW 264 cells (Figure 5). This observation was also similar when mammalian cells were grown under serum-depleted condition [16,18]. This is contrary to previous observations by other investigators, in which N^1 -acetylspermidine, in addition to spermidine, was the major form of effluxed polyamines from the cell [10,11,13,14]. Acetylation of polyamines has been proposed by many as one strategy employed by the cell to diminish the number of positive charges and to facilitate polyamine export from the cells [28,29]. In addition, acetylation has been proposed as a prerequisite to polyamine excretion [15,30]. The data presented here suggest otherwise, i.e. that acetylation of spermidine and spermine might have occurred in the cell, but the acetylated products were oxidized by the cytosolic polyamine oxidases to the final product, putrescine. This putrescine could then have been effluxed out by the RAW 264 cell into the culture medium. We have observed this phenomenon, i.e. markedly greater (or exclusively) putrescine and cadaverine export, in a variety of cell lines, including primary mouse macrophages, human T-lymphocytes, Reuber H35 rat hepatoma, ME180 human cervical carcinoma and A431 epidermoid carcinoma cells ([16,18], and results not shown). It would appear that, although ODC is believed to be a very highly regulated enzyme (controlling putrescine biosynthesis [3,31]), significant amounts of putrescine are continuously lost from the cell. Putrescine export might offer the cell another mechanism for carefully controlling the intracellular putrescine pool, or may indicate some extracellular function for the exported putrescine/cadaverine.

The export of polyamines by suspension cultures of *Neurospora* has been proposed to involve the non-specific simple diffusion of the amines through the membrane, regulated primarily by the concentration gradient [7,21]. However, the data illustrated in Figure 6 suggest that this may not be the case with the mammalian cells. As the intracellular putrescine concentrations increased, the rate of putrescine export decreased (Figure 6). If a non-saturable diffusional mechanism had been operable in the RAW 264 cells, one would have expected that an increase in the intracellular putrescine concentration would have been accompanied by a similar increase in the rate of putrescine efflux.

Moreover, the fact that cadaverine was never found to accumulate intracellularly, whereas its rate of export decreased as the cell numbers increased, suggested that the diamine may be selectively and efficiently transported out by the cell through a regulated system employing a membrane transport process. Our previous studies with the drugs verapamil and quinine {which have been shown to inhibit the multi-drug-resistance (MDR) P-glycoprotein export system [32,33]} showed that these agents were able to decrease markedly the levels of putrescine and cadaverine effluxed without significant changes in the levels of intracellular putrescine [19]. These observations suggest that diamine export in mammalian cells may occur through a facilitated-diffusion mechanism employing an integral membrane protein.

The decrease in the putrescine export rate seen as the cells grew toward confluency was primarily due to the presence of the effluxed putrescine present in the culture medium, and not to contact inhibition (Figures 8 and 9). This inhibition of putrescine export by extracellular putrescine required only very low levels of putrescine to be present in the culture medium ($K_i \sim 2 \mu\text{M}$). It is conceivable that, under normal physiological conditions, putrescine export by cells/tissues in an animal into the extracellular space occurs at very low levels, since the concentration of putrescine in animal serum is approx. 3 μM . The inhibition of putrescine export by extracellular putrescine also suggests the presence of an extracellular binding site for putrescine which might regulate a diamine-export protein. Such membrane binding sites for polyamines have recently been shown to exist as part of the NMDA receptor complex [34]. We are currently investigating the concentration-dependent regulation/inhibition of putrescine export, using a large series of naturally occurring and synthetic di- and poly-amines. Further characterization of the diamine-export process is currently also being performed using cell-free membrane vesicles, where the putrescine concentration can be tightly controlled, and the degree of saturability of this process can be quantified accurately.

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REFERENCES

- 1 Tabor, C. W. and Tabor, H. (1984) *Annu. Rev. Biochem.* **53**, 749–790
- 2 Pegg, A. E. (1986) *Biochem. J.* **234**, 249–262
- 3 Davis, R. H., Morris, D. R. and Coffino, P. (1992) *Microbiol. Rev.* **56**, 280–290
- 4 Pegg, A. E. (1988) *Cancer Res.* **48**, 759–774
- 5 Heby, O. and Persson, L. (1990) *Trends Biochem. Sci.* **15**, 153–158
- 6 Seiler, N. and Dezeure, F. (1990) *Int. J. Biochem.* **22**, 211–218
- 7 Davis, R. H. and Ristow, J. L. (1989) *Arch. Biochem. Biophys.* **271**, 315–322
- 8 Kashiwagi, K., Hosokawa, N., Furuchi, T., Kobayashi, H., Sasakawa, C., Yoshikawa, M. and Igarashi, K. (1990) *J. Biol. Chem.* **265**, 20893–20897
- 9 Kashiwagi, K., Yamaguchi, Y., Sakai, Y., Kobayashi, H. and Igarashi, K. (1990) *J. Biol. Chem.* **265**, 8387–8391
- 10 Pegg, A. E., Wechter, R., Pakala, R. and Bergeron, R. J. (1989) *J. Biol. Chem.* **264**, 11744–11749
- 11 Hyvonen, T. (1989) *Int. J. Biochem.* **21**, 313–316
- 12 Pegg, A. E., Pakala, R. and Bergeron, R. J. (1990) *Biochem. J.* **267**, 331–338
- 13 Porter, C. W., Ganis, B., Libby, P. R. and Bergeron, R. J. (1991) *Cancer Res.* **51**, 3715–3720
- 14 Wallace, H. M. and Keir, H. M. (1981) *Biochim. Biophys. Acta* **676**, 25–30
- 15 Wallace, H. M. and Keir, H. M. (1986) *FEBS Lett.* **194**, 60–63
- 16 Tjandrawinata, R. R., Hawel, L. and Byus, C. V. (1994) *J. Immunol.* **152**, 3039–3052
- 17 Hawel, L., Tjandrawinata, R. R. and Byus, C. V. (1994) *J. Biol. Chem.* **269**, 7412–7418
- 18 Hawel, L., Tjandrawinata, R. R. and Byus, C. V. (1994) *Biochim. Biophys. Acta* **1222**, 15–26
- 19 Tjandrawinata, R. R., Hawel, L. and Byus, C. V. (1994) *Biochem. Pharmacol.* in the press
- 20 Gahl, W. A. and Pitot, H. C. (1982) *Biochem. J.* **202**, 603–611

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- 21 Davis, R. H. (1990) *Biochem. Biophys. Res. Commun.* **172**, 199–205
- 22 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 23 Kabra, P. M., Lee, H. K., Lubich, W. P. and Marton, L. J. (1986) *J. Chromatogr.* **380**, 19–32
- 24 Gilbert, R. S., Gonzalez, G. G., Hawel, L. and Byus, C. V. (1991) *Anal. Biochem.* **199**, 86–92
- 25 Stein, W. D. (1986) *Transport and Diffusion across Cell Membranes*, Academic Press, San Diego
- 26 van Zoelen, E. J. (1991) *Crit. Rev. Oncog.* **2**, 311–333
- 27 Wieser, R. J., Renauer, D., Schafer, A., Heck, R., Engel, R., Schutz, S. and Oesch, F. (1990) *Environ. Health Perspect.* **88**, 251–253
- 28 Casero, R. A., Jr. and Pegg, A. E. (1993) *FASEB J.* **7**, 653–661
- 29 Seiler, N. (1987) *Can. J. Physiol. Pharmacol.* **65**, 2024–2035
- 30 Wallace, H. M. and Coleman, C. S. (1990) *Biochem. Soc. Trans.* **18**, 1091–1094
- 31 Kameji, T., Hayashi, S., Hoshino, K., Kakinuma, Y. and Igarashi, K. (1993) *Biochem. J.* **289**, 581–586
- 32 Lehnert, M., Dalton, W. S., Roe, D., Emerson, S. and Salmo, S. E. (1991) *Blood* **77**, 348–354
- 33 Lum, B. L., Gosland, M. P., Kaubisch, S. and Sikic, B. I. (1993) *Pharmacotherapy* **13**, 88–109
- 34 Williams, K., Romano, C., Dichter, M. A. and Molinoff, P. B. (1991) *Life Sci.* **48**, 469–498
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