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CHARACTERIZATION OF PUTRESCINE AND CADAVERINE EXPORT IN MAMMALIAN CELLS

A PHARMACOLOGICAL APPROACH

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Abstract—We characterized the mechanism(s) involved in the efflux of putrescine/cadaverine from cultured mammalian cells using various pharmacological agents. Verapamil and quinine inhibited putrescine and cadaverine export in monocytic-leukemic RAW 264 and H35 hepatoma cells in a concentration-dependent manner with an IC_{50} in the micromolar range. Verapamil, which inhibits L-type calcium channels, inhibited putrescine export, regardless of whether calcium was present in the extracellular medium or not. Furthermore, the export of putrescine in the absence of verapamil did not appear to depend upon extracellular calcium. Neither intracellular calcium, external sodium, changes in intracellular pH nor phosphorylation affected the levels of putrescine export independently from changes in intracellular putrescine levels. The data suggest that verapamil and quinine inhibit putrescine/cadaverine efflux from the cell by binding directly to an integral membrane protein.

Key words: polyamines; putrescine; cadaverine; verapamil; quinine; polyamine export

The polyamines putrescine, spermidine, and spermine are multivalent cations that, respectively, carry two, three, and four amine groups that are ionizable at physiological pH [1]. They are present ubiquitously in both prokaryotic as well as eukaryotic cells, and their accumulation inside the cell has been shown to be an essential event during proliferation and differentiation of both normal and neoplastic cells [2, 3]. Numerous studies have shown polyamine biosynthesis to be increased markedly following administration of various growth-promoting stimuli that induce the highly regulated enzyme ODC§ (EC 4.1.1.17) [2, 4].

In addition to alterations in putrescine/polyamine biosynthesis, the steady-state concentration of intracellular polyamines can be further regulated by changes in turnover (degradation), uptake, and export [5–7]. While a specific polyamine uptake system has been reasonably well characterized [8–10], the process involved in the export of polyamines from the cell remains poorly understood. In this regard, extracellular polyamines have been reported recently to possess two potential functions in the central nervous system requiring a process whereby their release from the cell could be regulated. A polyamine recognition site has been characterized on the NMDA–receptor complex, which increases the binding of open-channel blockers and increases NMDA-elicited currents in neurons [11]. These authors postulate that the polyamines may be selectively released from the neuronal cells in order to modulate the NMDA–receptor complex [11]. In addition, the polyamine agmatine has been shown to be locally synthesized in the brain and to function as an endogenous ligand/agonist at imidazoline receptors and as a ligand at α_2-adrenergic receptors [12]. These data are consistent with the hypothesis that the polyamines may function as neurotransmitters or neuromodulators, and thus would require a highly regulated transport system to cross the cell membrane.

We have reported previously that dividing and quiescent cultured mammalian cells, such as the macrophage-like RAW 264 cells and Reuber H35 hepatoma cells, constitutively export significant amounts of putrescine and cadaverine into the culture medium [13, 14]. This export process appeared to be selective for putrescine and cadaverine in that only a small amount of other polyamines (spermidine and N^1-acetylspermidine) was found in the culture medium. The net rate of constitutive putrescine export was markedly higher than even the highest stimulated rate of intracellular putrescine accumulation [13, 15], suggesting further that intracellular polyamine concentrations can be regulated, in part, by putrescine export. The export of putrescine was stimulated by a variety of agents including exogenous ornithine (a substrate for ODC), mitogens such as insulin, and differentiation factors such as bacterial LPS and interferon-γ [13–15]. Unlike the bacterial putrescine export system, which was *trans*-stimulated by ornithine [16], the cumulative data did not support a similar mechanism

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§ Abbreviations: BAPTA, 1, 2-bis (2-aminophenoxy)-ethane-*N*, *N*, *N*,′, *N*′-tetraacetic acid; LPS, lipopolysaccharide; MDR, multidrug resistance; NH_4Cl, ammonium chloride; NMDA, *N*-methyl-D-aspartate; ODC, ornithine decarboxylase; PKC, protein kinase C.

for putrescine export operating in mammalian cells [13–15]. Due to the potential importance of extracellular polyamines in the central nervous system [11, 12] and the clear role of putrescine export in regulating intracellular polyamine levels, we wished to characterize more fully in the RAW 264 and H35 cells the process involved in putrescine and cadaverine export.

A non-saturable, diffusional system has been implicated for putrescine export in suspension cultures of *Neurospora* [17]. However, the rate of putrescine export did not always correlate positively with changes in the intracellular putrescine concentration when comparing export rates between different stimuli and as putrescine levels changed with time [13–15]. Thus, it is conceivable that mammalian cells employ an integral membrane protein to export these diamines. Furthermore, there have been recent reports suggesting that putrescine export in mammalian cells may involve calcium, sodium, potassium, or even cytoplasmic alkalinization of the cells [18, 19]. To address these questions, a series of selective pharmacological agents known to alter the intracellular or extracellular concentration of these ions or inhibit other regulatory processes, such as protein phosphorylation (see below), was employed.

The MDR protein, P-glycoprotein, is a well-characterized transport protein involved in the export of various antineoplastic drugs. P-glycoprotein expression in multidrug resistance cell lines correlates with both the decrease in intracellular drug accumulation and the observed degree of drug resistance [20]. Using the MDR system as a working model or example of an export system, we followed a similar pharmacological approach to characterize putrescine/cadaverine efflux. Several compounds, originally described as calcium channel blockers or calmodulin inhibitors and found to inhibit the multidrug resistance protein, were investigated for similar effects upon diamine export [20–24]. In this regard, preliminary studies indicated that the drug verapamil, which inhibits the P-glycoprotein, also inhibited putrescine export in a similar manner [13, 15].

The potential involvement of extracellular or intracellular calcium in the putrescine export process was investigated by employing the L-type calcium channel blockers verapamil and nifedipine, the calcium ionophore A23187, the intracellular calcium chelator BAPTA, and calcium-free medium. The possible involvement of either sodium, potassium or cytoplasmic alkalinization in the mammalian putrescine export process was also investigated using quinine (a potassium channel blocker), NH_4Cl, monensin (a Na^+ ionophore), and ouabain [a (Na^+/K^+) ATPase inhibitor]. Furthermore, the possibilities of the regulation of the putrescine export system through phosphorylation were also addressed by using various protein kinase inhibitors such as staurosporine (a PKC inhibitor), trifluoroperazine (a calmodulin antagonist), and KN-62 (a selective calcium/calmodulin protein kinase II inhibitor).

To characterize selectively the putrescine export process using these various pharmacological agents or inhibitors, it was crucial that these compounds did not alter intracellular putrescine levels. Changes in intracellular putrescine have been observed to lead to alterations in the rate of putrescine efflux, especially in the presence of exogenous ornithine [13–15]. For this reason, intracellular polyamine levels were monitored carefully during all experiments with the various inhibitors so that the effects of these agents on putrescine and cadaverine export could be distinguished from changes in the intracellular levels. In addition, the concentration of the inhibitors and the length of time the cells were exposed were important parameters to monitor in an effort to minimize the nonspecific toxicity of the inhibitors to the cells.

MATERIALS AND METHODS

Chemicals and supplies. Dulbecco's modified Eagle's medium (DMEM), calcium-free DMEM, FBS, and all supplementary media antibiotics were obtained from Gibco BRL (Gaithersburg, MD). The Mycoplasma Detection Kit was purchased from Gen-Probe (San Diego, CA), while BM Cycline and Mycoplasma Removal Agent were purchased from Boehringer Mannheim (Indianapolis, IN) and ICN Biomedicals (Costa Mesa, CA), respectively. Bacterial LPS (from *Escherichia coli* serotype 0111:B4) and insulin were obtained from the Sigma Chemical Co. (St. Louis, MO), and ornithine was from Fluka Chemical (Ronkonkoma, NY). Dansyl chloride used to derivatize polyamines was obtained from Fluka, while Biorex-70 columns and C-18 hydrophobic columns were purchased from Bio-Rad (Richmond, CA) and Analytichem International (Harbor City, CA), respectively. Verapamil, quinine, A23187, BAPTA, nifedipine, monensin, and staurosporine were obtained from Sigma. KN-62 was purchased from LC Laboratories (Woburn, MA). Ouabain and trifluoroperazine were purchased from Calbiochem (San Diego, CA) and Biomol (Plymouth Meeting, PA), respectively. All other chemicals and supplies were obtained from Fisher Scientific (Fullerton, CA).

Cell culture. The mouse macrophage-like RAW 264 and rat Reuber H35 cells were maintained in DMEM (pH 7.4) supplemented with 10% FBS plus 50 IU/mL penicillin, 50 μg/mL streptomycin, and 10 μg/mL gentamycin. Cells were grown in 100 mm diameter plastic petri dishes at 37°, 5% CO_2 in humidified air. To rule out the presence of any contaminating mycoplasma, the cells were screened with the Mycoplasma Detection Test (as described by the manufacturer) and showed a negative result for mycoplasma. To further minimize the possible presence of mycoplasma, the RAW cells were grown in 3 cycles (1 cycle/week) of BM Cycline, and then in the Mycoplasma Removal Agent for 1 week according to instructions supplied by the manufacturer. Stock cultures of RAW 264 and H35 cells treated in this manner were retested with the Mycoplasma Detection Test and showed a negative result as well. The cells were frozen in liquid nitrogen and grown in culture for no longer than 2 weeks prior to each experiment.

The culture medium for the RAW 264 and H35 cells for all of the experiments described below was

changed from serum-containing to fresh serum-free medium 12 and 20 hr, respectively, prior to all experiments. Under this condition, RAW 264 and H35 cells remained highly viable (>95% as judged by trypan blue exclusion) throughout a 36 hr period (data not shown). At time zero, the medium was again changed to fresh serum-free medium (pre-warmed to 37° and gassed with 5% CO_2) with and without LPS, insulin, or ornithine. The cells were placed in serum-free medium to allow the cultures to slow their growth and enter stationary phase. In this manner, both cell lines could respond in a synchronous manner to LPS, insulin, or ornithine (see Results) [14, 15]. In addition, serum has been reported also to contain significant levels of diamine oxidase activity [25]. Such enzymatic activity markedly reduces the level of putrescine that can be measured in the culture medium. For the experiments involving calcium-free medium, this culture medium was supplemented with EGTA (0.5 mM) to completely chelate residual calcium.

Sample collection. Cells were washed rapidly with phosphate-buffered saline, 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, 500 μL of 0.5 N perchloric acid (PCA) was added. The cells were homogenized by sonication and, following further centrifugation at 10,000 *g*, the supernatant was subjected to intracellular polyamine and amino acid analyses, as described below. The precipitate was dissolved in 0.5 M NaOH for protein determination by the method of Bradford [26], using bovine serum albumin as a standard. All polyamine values were normalized to the amounts of acid-precipitable protein determined in this manner.

Determination of intracellular and extracellular polyamine levels. The intracellular polyamine analysis was performed using an HPLC procedure employing pre-column derivatization with dansyl chloride as described by Kabra *et al.* [27]. Acid-soluble supernatants (200–300 μL) were neutralized and reacted with 200 μL of 4 mg/mL of dansyl chloride in acetone for 15 min at 70°. The reacted samples were then prechromatographed on small C-18 hydrophobic columns, eluted with 1.5 mL methanol, and subsequently analyzed on HPLC.

Analysis of extracellular polyamines in culture medium was performed using a clean-up procedure prior to employing pre-column derivatization with dansyl chloride as described by Gilbert *et al.* [28]. Briefly, the culture medium sample was subjected to Biorex-70 chromatography, 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 N NH_4OH, and the extracellular polyamines were eluted with 3 mL of 4.0 N NH_4OH. The eluted samples were roto-evaporated to dryness, neutralized, and reacted with dansyl chloride to a final concentration of 2.0 mg/mL. The derivatized sample was then subjected to HPLC analysis. Using this procedure, putrescine, spermidine, spermine, cadaverine as well as all conjugated derivatives of polyamines, i.e. acetylputrescine, N^1- and N^8-acetylspermidine, and acetylspermine, could be quantified in 10 mL of culture medium with a limit of detectability of as low as 0.1 nmol/mg total cellular protein.

Concentration of drugs/inhibitors. The concentrations of agents used in all experiments reported in this paper were comparable to those found in the literature and referenced in Results. We minimized the length of time the cells were exposed in order to minimize the toxicity of the agents to the cells and to quantitate accurately the exported putrescine/cadaverine. Under all of the conditions (concentrations of drug and time of exposure) described in Results and in the figure legends, the RAW 264 and H35 cells remained highly viable throughout the experiments (>95%), as judged by trypan blue exclusion. A number of additional pharmacological agents employed proved to be toxic to the cells, as evidenced by trypan blue uptake and the appearance of spermine in the medium (data not shown).

RESULTS

Accumulation of putrescine and cadaverine in the culture media of RAW 264 and H35 cells. We reported previously that the efflux of putrescine and cadaverine is linear throughout 24 and 36 hr in RAW 264 and H35 cells, respectively, grown in serum-free medium [14, 15]. For the inhibitor studies described below, it was important to minimize the time the cells were exposed to these agents in order to minimize any nonspecific toxicity. It was also desirable to define experimental parameters whereby both the constitutive and stimulated export of putrescine could be investigated.

Quiescent, unstimulated RAW 264 and H35 cells selectively exported easily measurable amounts of putrescine and cadaverine into the serum-free culture medium constitutively throughout an 8 hr period (Fig. 1, A and B). When the cells were treated with exogenous ornithine (1 mM), the rate of putrescine export was increased over the 8 hr time course by factors of 1.5 and 3 compared with the controls in the RAW 264 and H35 cells, respectively. As reported previously, the addition of exogenous ornithine to either cell type, however, significantly reduced cadaverine effluxed to a value of approximately 0.2 nmol/mg protein [14]. Stimulation with either LPS (a macrophage differentiation factor in the RAW 264 cells) or insulin (a potent mitogen of H35 cells) did not change the rate of putrescine export during an 8 hr period (the stimulatory effect of LPS and insulin was observed between 8 and 12 hr [13, 15]. LPS, however, increased cadaverine export by RAW 264 cells between 4 and 8 hr of treatment (Fig. 1A). Very little spermidine (average = 0.3 nmol/mg protein) was exported by RAW 264 cells, and none at all by H35 cells, and no spermine was exported by either cell line. At zero time was cadaverine found to accumulate intracellularly to detectable levels, even when stimulated with either LPS or insulin. For all of the subsequent studies the cells were treated with the various agents for 4 or 8 hr, and the effects of these drugs upon the constitutive and stimulated export of putrescine and cadaverine, as well as the intracellular polyamine levels, were determined as described in Materials and Methods.

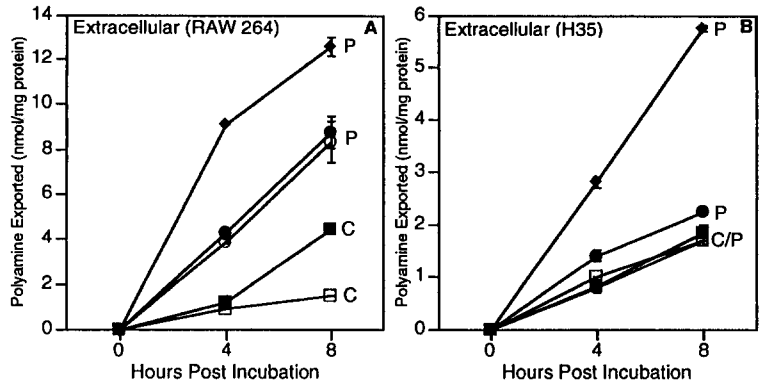

Fig. 1. Time-dependent alterations in extracellular polyamine accumulation in RAW 264 (A) and H35 (B) cells in the presence of exogenous ornithine, lipopolysaccharide (LPS), or insulin. RAW 264 and H35 cells were grown in serum-containing medium and received serum-free medium 12 hr prior to treatment. At time 0, the medium was again changed, and ornithine (1 mM), LPS for RAW 264 cells (1 μg/mL), or insulin for H35 cells (10^{-7} M) was added where indicated. Cells were then washed and harvested at the indicated times. The extracellular putrescine and cadaverine were determined as described in Materials and Methods and were represented in terms of nmol/mg protein. Each symbol represents the mean ± SEM of three determinations from three culture dishes. For many data points, the SEM was smaller than the size of the symbol as illustrated on the graphs. (For putrescine: ○ = control, ● = LPS or insulin, ◆ = ornithine; for cadaverine: □ = control, ■ = LPS or insulin.)

Effect of administration of verapamil on accumulation of intracellular and exported putrescine and cadaverine in both RAW 264 and H35 cells. It has been reported recently in bacterium that the polyamine export system involves putrescine–ornithine exchange through an antiporter system [16]. While previous studies indicated that the mammalian export process was not *trans*-stimulated by ornithine [13, 15], putrescine export has been postulated to involve some other positively charged ions such as sodium or calcium [18, 19]. The drug verapamil is an L-type calcium channel blocker and has been widely used to inhibit calcium influx into cells [29]. We used verapamil to investigate the hypothesis that the export of putrescine in mammalian cells may involve calcium as a regulatory factor in both RAW 264 and H35 cells (Figs. 2 and 3, respectively). Although concentrations of up to 2 μM verapamil did not change the level of putrescine export, verapamil at levels between 2 and 20 μM strongly inhibited the efflux of putrescine (Fig. 2A). The addition of LPS did not change the degree of putrescine export inhibition imposed by verapamil. The verapamil-mediated reduction in putrescine export seen between 2 and 20 μM was not accompanied by a reduction in intracellular putrescine accumulation in the RAW 264 cells (Fig. 2B). Thus, the inhibitory effect of this drug does not appear to be a secondary effect upon polyamine/putrescine biosynthesis or degradation. Intracellular spermidine and spermine levels were also not changed markedly by the administration of verapamil to the culture medium (Fig. 2C). Under the same conditions, verapamil also inhibited the export of cadaverine into the culture medium of RAW 264 cells (Fig. 2A).

To investigate further whether this inhibition was a phenomenon widely occurring in other mammalian cells, cultured Reuber H35 hepatoma cells were treated with verapamil in the presence of ornithine or insulin (Fig 3). In the control H35 cultures, verapamil at a concentration of 0.2 μM increased moderately the levels of putrescine and cadaverine export to levels 2- and 1.5-fold higher, respectively, than the basal/untreated values (Fig. 3A). However, at concentrations higher than 0.2 μM, verapamil markedly decreased the efflux of these diamines with a complete inhibition of export occurring at 20 μM verapamil. The steady-state intracellular putrescine levels, however, were not changed by the administration of verapamil at any of the levels employed. When diamine export was stimulated by insulin or ornithine (Fig. 3, B and C, respectively), administration of verapamil between 0.02 and 20 μM caused a concentration-dependent inhibition of both putrescine and cadaverine export. (Extracellular cadaverine accumulation in the H35 culture medium is inhibited completely by the presence of exogenous ornithine [14].) In both cases, the level of intracellular putrescine remained unchanged by the verapamil treatment. Again, the ability of verapamil to inhibit diamine efflux appeared to be independent of any effect upon polyamine biosynthesis/turnover since the intracellular levels did not change. In this regard, the Reuber H35 hepatoma cells appeared to be more sensitive to the inhibition of putrescine and cadaverine exports by verapamil than did the RAW 264 cells (Figs. 2 and 3).

Effect of exogenous calcium on the export of putrescine in RAW 264 cells. Verapamil has been reported to have pleiotropic effects upon the cell. In addition to inhibiting L-type calcium channels, verapamil has been reported to bind directly to the MDR-export protein [20, 21, 30], and to inhibit PKC

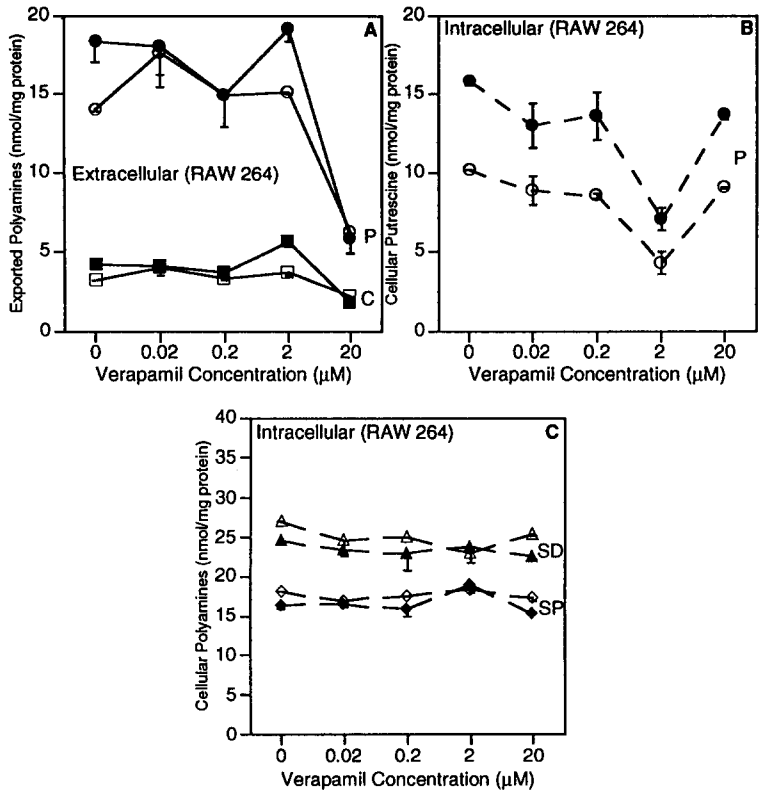

Fig. 2. Effect of verapamil on extracellular putrescine and cadaverine (A), intracellular putrescine (B), and intracellular spermidine and spermine (C) accumulation in RAW 264 cells in the presence/absence of LPS. RAW 264 cells were grown in serum-containing medium and received serum-free medium 12 hr prior to treatment. At time 0, a new serum-containing medium and verapamil at the concentrations indicated were added in the absence and in the presence of LPS (1 μM). Eight hours later, cells were harvested, and the intracellular and extracellular putrescine and extracellular cadaverine were determined as described in Materials and Methods. Each symbol represents the mean ± SEM of three determinations from three culture dishes. For many data points, the SEM was smaller than the size of the symbol as illustrated on the graphs. (Open symbol = control, filled symbol = LPS; ○,● = putrescine, □, ■ = cadaverine; △, ▲ = spermidine,; ◇, ◆ = spermine.)

in vitro with an IC_{50} of 0.5 mM [23, 31]. Thus, the reduction in export of putrescine and cadaverine in response to verapamil administration could indicate that (a) extracellular calcium might be involved in the export process, (b) verapamil inhibited PKC, which could regulate indirectly diamine export, or (c) verapamil interacted directly with the polyamine export system. To distinguish between these mechanisms, the role of extracellular calcium in polyamine export [mechanism (a)] was investigated by growing the RAW 264 cells in calcium-free medium.

Normally, DMEM culture medium contains 1.8 mM $CaCl_2$. When calcium was removed from the culture medium (see legend to Fig. 4 and Materials and Methods for details), putrescine export decreased only moderately (<35%) from the high rate of efflux observed in ornithine-stimulated RAW 264 cells. This decrease in putrescine export was not associated with a decrease in intracellular putrescine levels. Cadaverine was not detected in the culture medium since exogenous ornithine was added to the cells to maximize putrescine export. The data indicate that the presence of extracellular calcium was neither required nor necessary for putrescine export to occur, thus excluding the possibility of a putrescine–calcium exchange mechanism through an antiporter system. Nevertheless, extracellular calcium was capable of enhancing moderately the export process at concentrations higher than 0.1 mM (Fig. 4).

We next investigated whether the inhibitory action of verapamil on putrescine export was dependent upon the presence of extracellular calcium (Fig. 5, A and B). As seen previously (Figs. 3 and 4), in the presence of calcium, verapamil inhibited putrescine export between 2 and 20 μM. This inhibition of export by verapamil also occurred even when extracellular calcium was not present in the culture medium. No significant differences in intracellular putrescine levels were observed in the verapamil-treated cells incubated in the presence or in the absence of extracellular calcium. The data suggest that the inhibitory effect of verapamil upon

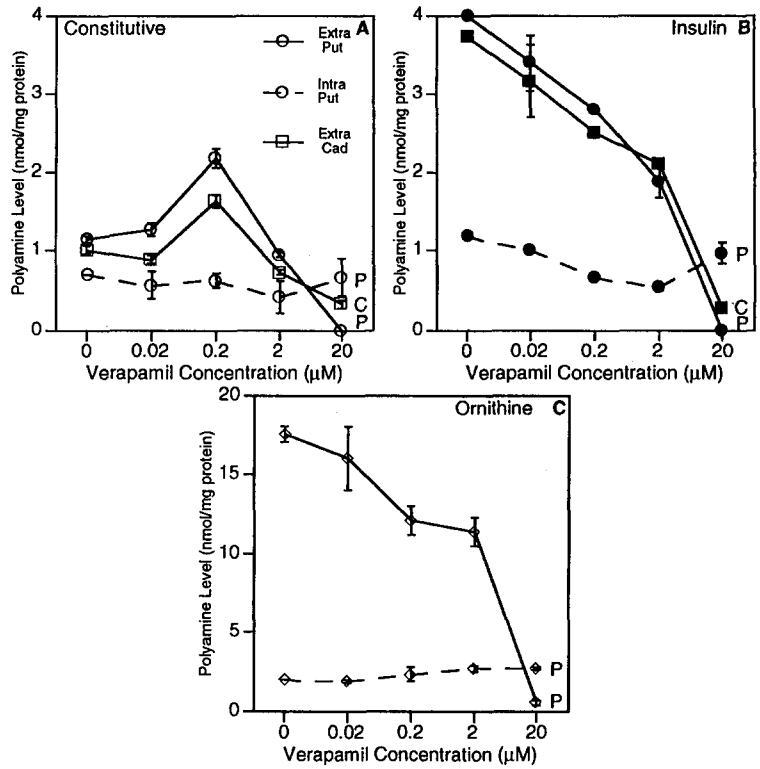

Fig. 3. Effect of verapamil on extracellular putrescine and cadaverine and intracellular putrescine accumulation in H35 cells in control cultures (A), and in the presence of insulin (B) or ornithine (C). H35 cells were grown in serum-containing medium and received serum-free medium 12 hr prior to treatment. At time 0, a new serum-containing medium and verapamil at the concentrations indicated were added in the presence of the indicated treatment. Eight hours later, cells were harvested, and the intracellular and extracellular putrescine and extracellular cadaverine were determined as described in Materials and Methods. Each symbol represents the mean ± SEM of three determinations from three culture dishes. For many data points, the SEM was smaller than the size of the symbol as illustrated on the graphs. (Solid line = extracellular, dashed line = intracellular; ○, ●, ◇ = putrescine in the control, insulin-, and ornithine-treated cells, respectively; □, ■ = cadaverine in control and insulin-treated cells, respectively.)

polyamine efflux did not require extracellular calcium.

Effects of agents capable of altering intracellular calcium levels upon accumulation of intracellular and exported putrescine in RAW 264 cells. Although the studies with calcium-free medium suggested that *extracellular* calcium *per se* was not required for the diamine export process to occur, it was conceivable that *intracellular* calcium may play a regulatory role in the export process. To test this hypothesis, several agents capable of modulating the intracellular calcium level were screened for their ability to increase or inhibit putrescine export (without affecting intracellular putrescine accumulation). The agents used for this purpose (shown in Table 1) were A23187 (a calcium ionophore that increases intracellular calcium levels [32]), BAPTA (an agent permeable to cell membranes capable of chelating intracellular calcium [33]), and nifedipine (a dihydropyridine derivative capable of inhibiting L-type calcium channels at a site different from that of verapamil [29]).

The calcium ionophore A23187 did not alter either the constitutive (control) export of putrescine or the intracellular level. However, A23187, in cells treated with ornithine, actually led to nearly a 2-fold increase in putrescine export. This increase in putrescine export brought about by the calcium ionophore, however, was accompanied by a concomitant increase in intracellular putrescine, suggesting that the rise in export was secondary to an increase in polyamine biosynthesis. Thus, A23187 did not appear to directly modulate the putrescine export system. BAPTA, a calcium chelator, did not alter either the efflux of putrescine from the cell or the intracellular concentration of putrescine. These results suggest that intracellular calcium does not play a major role in the putrescine export process.

If the inhibition of putrescine export brought about by verapamil was due to inhibition of a calcium channel, then another inhibitor would be expected to cause a similar effect. Nifedipine has been reported to inhibit L-type calcium channels by binding to the channel at a site different from that of verapamil [29]. Nifedipine administration did not alter the constitutive export of putrescine in either

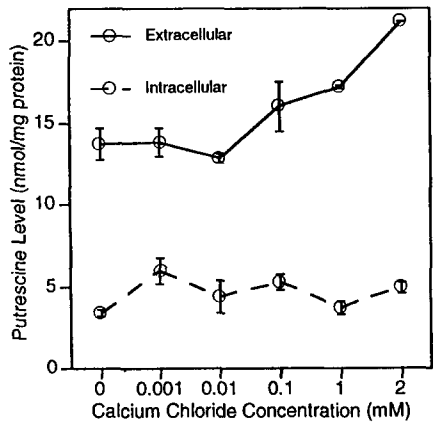

Fig. 4. Effect of extracellular calcium chloride upon extracellular and intracellular putrescine in RAW 264 cells. RAW 264 cells were grown in serum-containing medium and received serum-free medium 12 hr prior to treatment. Four hours prior to time 0, cells received exogenous ornithine (1.0 mM) to maximize the putrescine export. At time 0, serum- and calcium-free medium, ornithine (1.0 mM) and exogenous $CaCl_2$ at the concentrations indicated were added. Four hours later, cells were harvested, and the intracellular and extracellular putrescine and extracellular cadaverine were determined as described in Materials and Methods. In cultures not receiving exogenous calcium, EGTA (0.5 mM) was added to chelate traces of calcium that might be present in the commercial medium. Each symbol represents the mean ± SEM of three determinations from three culture dishes. For many data points, the SEM was smaller than the size of the symbol as illustrated on the graphs. (Solid line = extracellular, dashed line = intracellular.)

the RAW 264 or H35 cells (Table 1). In the ornithine-stimulated cells, 10 μM nifedipine did inhibit the efflux of putrescine. However, unlike verapamil, the inhibition of putrescine export by nifedipine was accompanied by a concomitant decrease in the accumulation of intracellular putrescine. It was conceivable that nifedipine was moderately toxic to the cells at this concentration or was inhibiting putrescine biosynthesis. However, nifedipine did appear to inhibit putrescine export specifically. No effect of A23187, BAPTA, or nifedipine was seen on constitutive cadaverine export (in the presence of ornithine, cadaverine is not detected in the culture medium [14]).

Effect of administration of quinine on exported putrescine and intracellular polyamines in both RAW 264 and H35 cells. In MDR cell lines, quinine, in addition to verapamil, has been found to inhibit the efflux of antineoplastic drugs facilitated by P-glycoprotein [24, 34]. We wished to determine whether quinine (a potassium channel blocker) possessed a similar inhibitory effect on the export of putrescine and cadaverine in RAW 264 and H35 cells. In the control cultures of RAW 264 cells, quinine decreased the export of putrescine concentration dependently (Fig. 6A). The export of cadaverine by RAW 264 cells was also inhibited at concentrations of quinine up to 25 μM (Fig. 6B). At a quinine concentration of 50 μM, the level of effluxed putrescine was approximately 3-fold lower than that of the untreated cultures. When LPS was added to stimulate putrescine export, quinine administration also led to a marked inhibition of putrescine efflux. This quinine-mediated reduction in the efflux of putrescine was not accompanied by any alteration in the level of intracellular putrescine up to 25 μM (Fig. 6B) or the level of intracellular spermidine and spermine (Fig. 6C). However, 50 μM

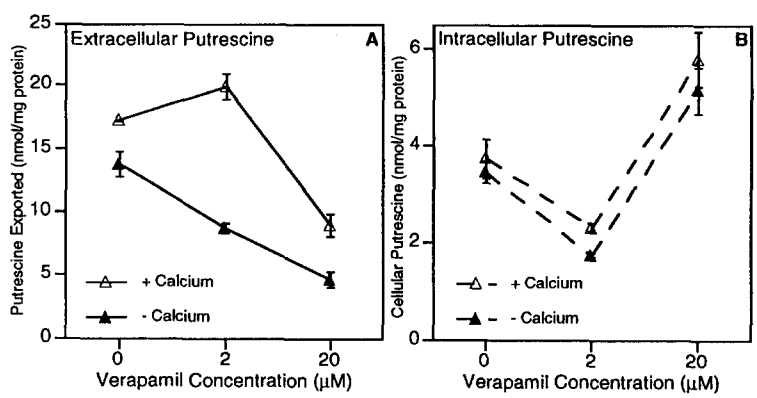

Fig. 5. Effect of verapamil on exported and intracellular putrescine accumulation in RAW 264 cells grown in calcium-free medium. RAW 264 cells were grown in serum-containing medium and received serum-free medium 12 hr prior to treatment. Two hours prior to time 0, cells received exogenous ornithine (1 mM). At time 0, verapamil at the indicated concentrations was added to a new calcium-free medium (no serum added). Four hours later, cells were harvested, and the intracellular and extracellular putrescine and extracellular cadaverine were determined as described in Materials and Methods. In cultures not receiving exogenous calcium, EGTA (0.5 mM) was added to chelate traces of calcium that might be present in the commercial medium. Each symbol represents the mean ± SEM of three determinations from three culture dishes. For many data points, the SEM was smaller than the size of the symbol as illustrated on the graphs.

Table 1. Effect of drugs capable of affecting cellular calcium levels on intracellular and exported putrescine

	RAW 264 cells		H35 cells	
	Intracellular (nmol/mg protein)	Exported (nmol/mg protein)	Intracellular (nmol/mg protein)	Exported (nmol/mg protein)
Control (constitutive)	4.14 ± 0.30	7.83 ± 0.20		
A23187 (0.05 μM)	4.85 ± 0.27	8.83 ± 0.05		
A23187 (0.25 μM)	4.87 ± 0.06	7.00 ± 0.29		
Ornithine	4.64 ± 0.20	7.82 ± 0.25		
A23187 (0.05 μM) + Orn	7.07 ± 0.40	10.73 ± 0.85		
A23187 (0.25 μM) + Orn	9.91 ± 0.30	13.25 ± 0.80		
Control (constitutive)	2.70 ± 0.30	8.09 ± 0.97	0.73 ± 0.02	1.71 ± 0.04
BAPTA (10 μM)	2.52 ± 0.10	7.40 ± 0.10	0.53 ± 0.08	1.73 ± 0.02
Ornithine	5.77 ± 0.60	16.86 ± 0.65	1.61 ± 0.21	16.34 ± 0.16
BAPTA (10 μM) + Orn	4.86 ± 0.32	17.86 ± 1.20	1.25 ± 0.34	14.01 ± 0.50
Control (constitutive)	2.70 ± 0.30	8.09 ± 0.97	0.73 ± 0.02	1.71 ± 0.04
Nifedipine (1 μM)	3.17 ± 0.18	7.98 ± 0.17		
Nifedipine (10 μM)	1.28 ± 0.08	7.89 ± 0.96	0.86 ± 0.02	2.30 ± 0.26
Ornithine	5.77 ± 0.60	16.86 ± 0.65	1.16 ± 0.21	16.34 ± 0.16
Nifedipine (1 μM) + Orn	4.98 ± 0.37	15.52 ± 0.58		
Nifedipine (10 μM) + Orn	2.38 ± 0.11	12.72 ± 0.44	1.03 ± 0.14	12.70 ± 0.91

The RAW 264 and H35 cells were grown in serum-containing medium until 50% confluency was reached. Twelve (RAW 264) and twenty (H35) hours prior to experiments, the culture medium was replaced with serum-free medium to allow the cells to become semi-quiescent. The medium was again changed to a new serum-free medium at time 0, and all treatments at the indicated concentrations together with exogenous ornithine (1 mM) were administered to the culture medium. Four (A23187) or six (BAPTA and nifedipine) hours later, cells were harvested, and the intracellular and extracellular putrescine were analyzed as described in Materials and Methods. Data are the means ± SEM of three determinations from three culture dishes.

quinine did lead to a 40% reduction in intracellular putrescine in the LPS-stimulated cells only.

A similar reduction in putrescine export brought about by quinine was seen also in Reuber H35 rat hepatoma cells (Fig. 7). Although this reduction was moderate in the control cultures (Fig. 7A), the inhibitory action of quinine on putrescine and cadaverine efflux was more pronounced in the presence of insulin and ornithine (Fig. 7, B and C). In either case, no significant change in intracellular putrescine concentration was seen in response to quinine treatment in these cells.

Effect of alkalinization of the cytoplasm on intracellular and exported putrescine in RAW 264 cells. It has been reported previously by Grillo and co-workers [19] that cellular alkalinization promotes the release of polyamines from human lymphocytes and L1210 cells. These investigators also reported that ouabain [a (Na^+/K^+)ATPase inhibitor] enhanced the accumulation of extracellular polyamines, primarily in the form of spermidine, from human lymphocytes [18]. Intracellular alkalinization has also been shown to increase ornithine decarboxylase activity 5-fold above controls in Reuber H35 cells [35]. We wished to determine whether a *decrease* in intracellular H^+ concentration through alkalinization of the cytoplasm with NH_4Cl or the use of monensin (a Na^+ ionophore that is capable of artificially exchanging Na^+ for H^+) or an *increase* in Na^+ concentration (using ouabain) had any effect upon polyamine efflux (Table 2).

While NH_4Cl did not alter the constitutive level of putrescine export, a 35% stimulation of putrescine efflux in the presence of LPS was seen as a result of NH_4Cl treatment. NH_4Cl did not change the level of intracellular putrescine accumulated in the presence or absence of LPS. Another method of cytoplasmic alkalinization using monensin was similarly unable to alter the export of putrescine. At 25 μM, ouabain (which increased cellular sodium levels) stimulated putrescine biosynthesis, and not the export, primarily when ornithine was present concomitantly. The data suggest that neither cytoplasmic H^+ nor Na^+ concentration has a minimal effect upon putrescine efflux.

Effect of protein kinase inhibitors on intracellular and exported putrescine levels in RAW 264 cells. As stated previously, verapamil could be inhibiting putrescine and cadaverine export through the known ability of this drug to inhibit PKC [mechanism (b)] [23, 31]. Some investigators have proposed that verapamil inhibits the MDR export protein indirectly by inhibiting its phosphorylation by PKC [23]. Earlier studies from various laboratories have shown also that staurosporine (a PKC inhibitor), trifluoroperazine (a calmodulin antagonist), and KN-62 (a Ca^{2+}/calmodulin-dependent protein kinase II inhibitor) decrease the phosphorylation of P-glycoprotein in MDR cell lines, resulting in an increase in the retention of several antineoplastic drugs intracellularly [22, 36, 37]. Following this approach, we tested if these inhibitors had a similar effect on the putrescine efflux system.

Staurosporine increased the level of putrescine exported in the absence and presence of exogenous

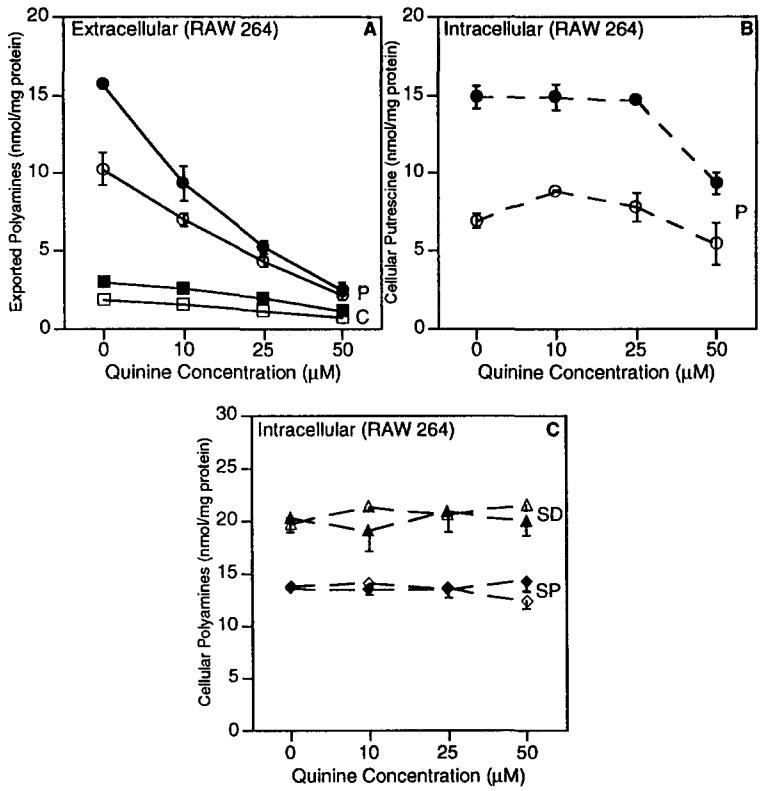

Fig. 6. Effect of quinine on extracellular putrescine and cadaverine (A), intracellular putrescine (B), and intracellular spermidine and spermine (C) accumulation in RAW 264 cells in the presence/absence of LPS. RAW 264 cells were grown in serum-containing medium and received serum-free medium 12 hr prior to treatment. At time 0, a new serum-containing medium and quinine at the concentrations indicated were added in the absence and in the presence of LPS (1 μM). Eight hours later, cells were harvested, and the intracellular and extracellular putrescine and extracellular cadaverine were determined as described in Materials and Methods. Each symbol represents the mean ± SEM of three determinations from three culture dishes. For many data points, the SEM was smaller than the size of the symbol as illustrated on the graphs. (Open symbols = control, filled symbols = LPS; ○, ● = putrescine, □, ■ = cadaverine; △, ▲ = spermidine; ◇, ◆ = spermine.)

ornithine (Table 3). However, this stimulation of putrescine export was accompanied by a similar increase in the intracellular putrescine level. KN-62 also caused a measurable increase in both intracellular and exported putrescine when the cells were treated with ornithine. Trifluoroperazine, a calmodulin inhibitor, slightly decreased intracellular and effluxed putrescine in the presence of exogenous ornithine. No changes in intracellular spermidine and spermine were seen as a result of administration of any of these agents (data not shown). These protein kinase inhibitors, therefore, did not appear to modulate the putrescine export system independently from changes in intracellular putrescine levels.

DISCUSSION

In cultures of *Neurospora*, the export of polyamines has been reported to involve a simple non-saturable, diffusional process directly proportional in rate to the difference between the intracellular and extracellular concentration of the polyamine [17]. However, the data presented here suggest that putrescine and cadaverine export do not involve simple diffusion through the lipid membrane. In the experiments employing two different cell lines of different origins, the export of putrescine and cadaverine was inhibited by verapamil and quinine. This inhibition was not associated with changes in the intracellular putrescine concentration (Figs. 2, 3, 6 and 7). The rate at which a small molecule diffuses across a membrane is determined primarily by the concentration gradient. If a simple diffusion mechanism were responsible for the efflux of putrescine, the decrease of putrescine export brought about by verapamil and quinine would have been accompanied by a similar reduction in the intracellular putrescine concentrations. Since the concentration gradient for putrescine and cadaverine was not changed by verapamil or quinine, it is difficult to hypothesize the manner in which these agents might alter other parameters important in the regulation of simple diffusion through the cell membrane (such as the area and thickness of the membrane or the oil/water partition coefficient of putrescine and cadaverine). For this reason and the observed lack

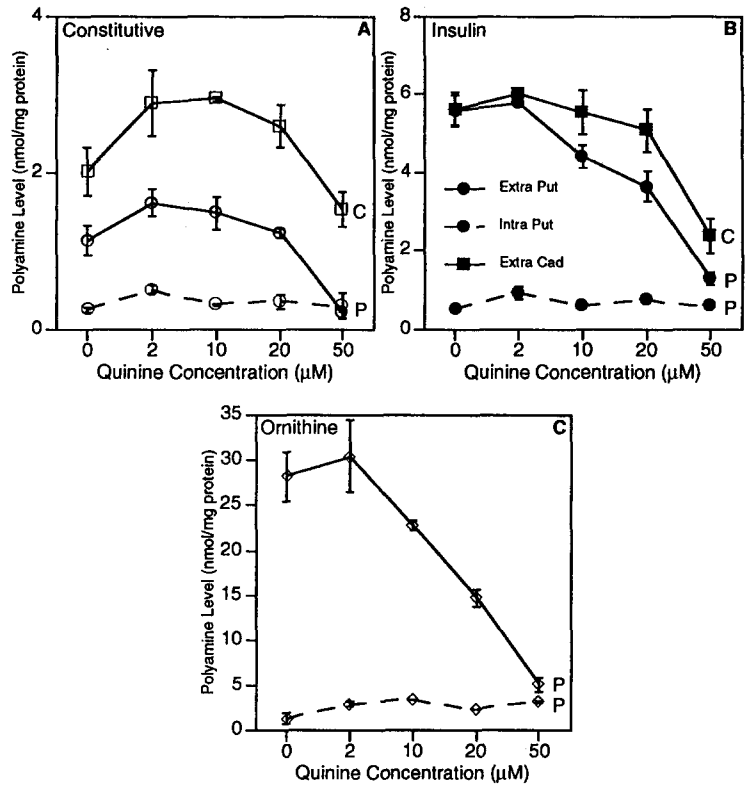

Fig. 7. Effect of quinine on extracellular putrescine and cadaverine and intracellular putrescine accumulation in H35 cells in control cultures (A), and in the presence of insulin (B) or ornithine (C). H35 cells were grown in serum-containing medium and received serum-free medium 12 hr prior to treatment. At time 0, a new serum-containing medium and quinine at the concentrations indicated were added in the presence of the indicated treatment. Eight hours later, cells were harvested, and the intracellular and extracellular putrescine and extracellular cadaverine were determined as described in Materials and Methods. Each symbol represents the mean ± SEM of three determinations from three culture dishes. For many data points, the SEM was smaller than the size of the symbol as illustrated on the graphs. (Solid line = extracellular, dashed line = intracellular; ○, ●, ◇ = putrescine in the control, insulin-, and ornithine-treated cells, respectively; □, ■ = cadaverine in control and insulin-treated cells, respectively.)

of correlation between putrescine export rates and intracellular putrescine levels described previously [13, 15], the evidence suggests that putrescine and cadaverine are exported by a facilitative diffusion mechanism involving an integral membrane protein(s).

The observed reduction of putrescine export brought about by verapamil suggested that extracellular calcium might be involved in the putrescine export process. The idea that putrescine–calcium exchange could have occurred through a bidirectional electronegative antiporter system was an attractive hypothesis. However, this hypothesis proved incorrect since putrescine export occurred even when medium (extracellular) calcium was not present (Fig. 4). In addition, the inhibitory action of verapamil on putrescine export was not dependent upon the presence of extracellular calcium (Fig. 5). It was also interesting to speculate that intracellular calcium was involved in the export process, perhaps involving a displacement/exchange mechanism between free intracellular calcium and bound putrescine or cadaverine. However, experiments employing the calcium ionophore A23187 and the calcium chelator BAPTA suggested otherwise (Table 1). For example, if intracellular calcium were capable of displacing bound putrescine inside the cell (thus allowing it to be available for export), the level of putrescine export would have been increased and decreased following administration of A23187 and BAPTA, respectively. A23187 has been reported to increase polyamine export in stimulated lymphocytes and L1210 cells [18, 19]. However, stimulation of ODC, spermidine/spermine N^1-acetyltransferase (SAT), and DNA synthesis has also been shown to occur following A23187 treatment [38]. The increase in polyamine export in lymphocytes, therefore, may have been due to increased polyamine concentrations in response to accelerated polyamine biosynthesis. We postulate that polyamine efflux is increased by a similar mechanism, which could have led to increased export in a manner similar to the effects seen in the RAW 264 cells after incubation with A23187 and ornithine (Table 1).

Table 2. Effect of alkalinization of the cytoplasm on intracellular and exported putrescine in RAW 264 cells

	Intracellular (nmol/mg protein)	Exported (nmol/mg protein)
Control (constitutive)	3.69 ± 0.09	7.08 ± 0.50
NH_4Cl (20 mM)	2.42 ± 0.24	6.56 ± 0.24
LPS	6.85 ± 0.24	10.76 ± 1.63
NH_4Cl (20 mM) + LPS	6.87 ± 0.01	14.19 ± 0.06
Control (constitutive)	4.14 ± 0.30	7.83 ± 0.20
Monensin (0.5 μM)	4.56 ± 0.27	5.27 ± 0.57
Monensin (2.5 μM)	6.85 ± 0.12	6.56 ± 0.61
Ornithine	4.64 ± 0.20	7.82 ± 0.25
Monensin (0.5 μM) + Orn	6.85 ± 0.12	7.10 ± 0.61
Monensin (2.5 μM) + Orn	7.52 ± 0.11	9.97 ± 0.58
Control (constitutive)	1.02 ± 0.07	2.31 ± 0.16
Ouabain (25 μM)	1.58 ± 0.84	2.34 ± 0.20
Ouabain (50 μM)	1.49 ± 0.43	2.59 ± 0.74
Ouabain (75 μM)	0.96 ± 0.03	2.25 ± 0.06
Ornithine	1.97 ± 0.25	3.39 ± 0.57
Ouabain (25 μM) + Orn	3.20 ± 0.50	3.73 ± 0.15
Ouabain (50 μM) + Orn	1.40 ± 0.16	3.26 ± 0.12
Ouabain (75 μM) + Orn	1.04 ± 0.04	3.30 ± 0.15

The RAW 264 cells were grown in serum-containing medium until 50% confluency was reached. Twelve hours prior to experiments, the culture medium was replaced with serum-free medium to allow the cells to become semi-quiescent. The medium was again changed to a new serum-free medium at time 0, and all treatments at the indicated concentrations together with LPS (1 μg/mL) or ornithine (1 mM) were administered to the culture medium. Four (monensin and ouabain) or eight (NH_4Cl) hours later, cells were harvested, and the intracellular and extracellular putrescine were analyzed as described in Materials and Methods. Data are the means ± SEM of three determinations from three culture dishes.

Table 3. Effect of protein kinase inhibitors on intracellular and exported putrescine in RAW 264 cells

	Intracellular (nmol/mg protein)	Exported (nmol/mg protein)
Control (constitutive)	2.70 ± 0.30	8.09 ± 0.97
Staurosporine (1 μM)	5.65 ± 0.82	12.73 ± 1.41
Staurosporine (5 nM)	7.54 ± 0.81	13.48 ± 0.52
Ornithine	5.77 ± 0.60	16.86 ± 0.65
Staurosporine (1 nM) + Orn	13.35 ± 1.47	26.75 ± 2.93
Staurosporine (5 nM) + Orn	18.25 ± 2.71	29.92 ± 2.58
Control (constitutive)	3.26 ± 0.50	6.38 ± 0.15
Trifluoroperazine (10 μM)	2.42 ± 0.14	5.71 ± 0.167
Ornithine	5.70 ± 0.37	21.98 ± 0.25
Trifluoroperazine (10 μM) + Orn	3.43 ± 0.23	17.01 ± 1.70
Control (constitutive)	3.26 ± 0.50	6.38 ± 0.15
KN-62 (10 μM)	3.72 ± 0.06	6.86 ± 0.53
Ornithine	5.70 ± 0.37	21.98 ± 0.25
KN-62 (10 μM) + Orn	9.77 ± 0.94	25.25 ± 2.87

The RAW 264 cells were grown in serum-containing medium until 50% confluency was reached. Twelve hours prior to experiments, the culture medium was replaced with serum-free medium to allow the cells to become semi-quiescent. The medium was again changed to a new serum-free medium at time 0, and all treatments at the indicated concentrations together with LPS (1 μg/mL) or ornithine (1 mM) were administered to the culture medium. Six hours later, cells were harvested, and the intracellular and extracellular putrescine were analyzed as described in Materials and Methods. Data are the means ± SEM of three determinations from three culture dishes.

The potential involvement of intracellular sodium and alkalinization upon the putrescine export process was investigated using ouabain, NH_4Cl and monensin (Table 2). Although NH_4Cl caused a 30–35% increase in putrescine export in the presence of LPS, other methods of cytoplasmic alkalinization (using monensin) were without effect on putrescine export both in the absence or presence of ornithine. Monensin, however, did cause a marked increase in intracellular (biosynthesis) and a slight increase in exported putrescine. Monensin, in addition to increasing the transport of sodium into the cells (in exchange for a proton), has been reported to cause a rise in cytosolic calcium [39], which partially explains the observed increase in putrescine biosynthesis in RAW 264 cells following addition of this agent. Cytoplasmic alkalinization employing NH_4Cl has been reported to stimulate ODC activity [35], and to increase both cellular sodium (through the Na^+/H^+ exchanger [40, 41]) and free calcium (which originates from intracellular storage [42]). However, we did not see an increase in putrescine biosynthesis in response to NH_4Cl administration. The data presented here further support the hypothesis that intracellular calcium and sodium do not participate directly in the putrescine export process.

Administration of staurosporine and KN-62 in the presence of ornithine led to increases in intracellular and exported putrescine in RAW 264 cells (Table 3). Staurosporine has been reported to induce ODC activity in the mouse epidermis [43, 44], which may partially explain the observed increase in putrescine biosynthesis seen in RAW 264 cells following administration of this inhibitor of PKC. However, the mechanism by which KN-62 induces an increase in intracellular and exported putrescine is currently unknown. It has been shown previously that calmodulin antagonists, such as trifluoroperazine and W-7, inhibit the serum-stimulated induction of ODC in a dose-dependent manner [45]. Thus, in response to trifluoroperazine treatment (Table 3), the reduction in putrescine biosynthesis, and therefore putrescine export, could be due to an inhibition of ODC activity in the RAW 264 cells.

The inhibition in putrescine export by verapamil and quinine was independent of changes in intracellular putrescine concentration in both RAW 264 and H35 cells (Figs. 2, 3, 6, and 7). While the effect of verapamil did not appear to involve extracellular calcium or inhibition of PKC as described above, the possibility remains that verapamil binds to the putrescine export system and inhibits it directly in a manner similar to that observed for the MDR-export protein [20, 21, 30]. While quinine has also been reported to inhibit export by the P-glycoprotein in a manner similar to verapamil, there is currently no reported evidence to show direct binding of quinine to the P-glycoprotein. However, such binding has been postulated to occur since quinine (a quinoline derivative) and verapamil (a phenylalkylamine derivative) are both lipid soluble at physiologic pH [46], due to the presence of lipophilic aromatic rings in both molecules [47] and share a number of other common structural features [47]. Thus, quinine could also bind to the putrescine export system leading to an inhibition of transport.

We are currently attempting to further characterize putrescine/cadaverine export using inside-out cell-free vesicles where the concentration of diamine can be controlled accurately and the quantification of its transport across the membrane can be performed precisely. Since no agents studied here selectively affected the export of putrescine relative to cadaverine, it cannot be determined whether there is a unique export process for cadaverine distinct from putrescine. Using this vesicle system, measurements of such specificity, saturability, and other kinetic parameters, such as V_{max} and K_m, could be determined.

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