Polyamine Replacement by Magnesium Ions in BHK-21/C13 Cells

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Cultures of BHK-21/C13 cells, whose growth was inhibited by deprivation of serum, were stimulated to grow by addition of serum to the culture medium. Addition of MgCl_2 to the medium, to increase the concentration of Mg^{2+} ions by 15 mM, 30 min before addition of serum, had no effect on the stimulation of cell growth, but inhibited the accumulation of cellular spermidine, so that the spermidine/spermine molar ratio was lower in these cultures than in cultures that had received no additional cations. The increase in the activity of ornithine decarboxylase that occurs 4–5 h after serum 'step-up' was substantially diminished by increasing the concentration of Mg^{2+} ions, but not of Na^+ or K^+ ions, in the medium by 30 mM, 30 min before addition of serum, and this inhibition was maintained for at least 24 h. Methylglyoxal bis(guanylhydrazone), added to serum-deprived cultures to a concentration of 20 μM , 30 min before addition of serum, severely inhibited the increase in cell growth. The inhibitory effects of the drug were prevented by simultaneous addition of spermidine to the medium (to 100 μM), and were partly prevented by the simultaneous addition of Mg^{2+} ions (to 30 mM). Mg^{2+} ions were particularly effective in overcoming the inhibitory effect of methylglyoxal bis(guanylhydrazone) on the synthesis of DNA. Thus although a certain lack of specificity for cations exists in BHK-21/C13 cells, in that Mg^{2+} ions can be substituted for polyamines, particularly spermidine, to some extent, there are cellular processes for which the requirement for polyamines as cations is specific.

Intracellular concentrations of putrescine, spermidine and spermine are closely related to the rate of cell proliferation (Bachrach, 1973), rapidly growing cells having a higher content of polyamines than non-proliferating cells (Heby *et al.*, 1975*b*) and a higher spermidine/spermine molar ratio (Heby *et al.*, 1975*a*). An unambiguous role for the polyamines *in vivo* has yet to be determined, for, although these amines affect the characteristics of a large variety of biochemical processes *in vitro* (Tabor & Tabor, 1976), their effects can usually be simulated, at least to some extent, by Mg^{2+} , albeit at considerably higher concentrations than required with polyamines (Konecki *et al.*, 1975; Cohen & Zalik, 1978).

As the polyamines carry a net positive charge at physiological pH, their general function is probably to serve as biological cations. In this respect they resemble Mg^{2+} ions (Rubin, 1976); however, it is difficult to determine whether polyamines or Mg^{2+} ions are the effective cations for any given system *in vivo*, because of the ubiquitous nature of each of these cations in cells.

We have used 'resting' cultures of BHK-21/C13 cells, whose growth was limited by deprivation of serum, to compare the requirements for polyamines and Mg^{2+} ions when cell growth rate was increased

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

by addition of serum to the culture medium (Bürk, 1970; Howard *et al.*, 1974). We compared the effectiveness of Mg^{2+} ions and spermidine in overcoming the shortage of cellular polyamines that results from inhibition of cell growth rate by the presence of methylglyoxal bis(guanylhydrazone) {1,1'-(methylethanediyldiene)dinitrilo}diguanidine (Heby *et al.*, 1977; Melvin & Keir, 1978*b*; Rupniak & Paul, 1978).

Experimental

Materials

DL-[1- ^{14}C]Ornithine hydrochloride (58 mCi/mmol) and [6- ^3H]thymidine (21–28 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, dansyl chloride, DL-dithiothreitol, Hepes base and hypoxanthine were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Methylglyoxal bis(guanylhydrazone) dihydrochloride monohydrate was from Aldrich Chemical Co., Gillingham, Dorset, U.K., and silica-gel 60 t.l.c. plates (20 cm $\times$ 20 cm) were from Merck, Darmstadt, Germany. Tissue-culture materials, including horse serum screened for mycoplasma, were from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.

Cell culture

BHK-21/C13 cells (McPherson & Stoker, 1962) were grown routinely in monolayer cultures at 37°C in a 5% CO₂ atmosphere in Dulbecco's modification of Eagle's medium (Dulbecco & Freeman, 1959) containing 0.1 mM-hypoxanthine and 10% (v/v) horse serum. This medium contained 0.8 mM-Mg²⁺, 140 mM-Na⁺ and 5 mM-K⁺ ions.

Determination of protein, RNA, DNA and polyamines

Cells were grown in 2.24-litre roller bottles until about two generations short of confluence. The cells were harvested and dispensed into 90mm-diameter vented plastic dishes at a density of 0.9×10^6 cells per dish, in Dulbecco's medium containing 0.1 mM-hypoxanthine and 10% (v/v) horse serum. After incubation at 37°C in a 5% CO₂ atmosphere for 24 h, the cell sheet was washed with medium containing 1% (v/v) serum, and the cells were incubated for 4–5 days in this medium with one further change of it (after 24 h). The cells were 'stepped-up' by replacing the medium by fresh medium containing 1% (v/v) serum, and cation if required [which had been dissolved previously in 0.9% (w/v) NaCl and sterilized by filtration]. After 30 min, serum was added to the dishes to a final concentration of 10% (v/v). The cells were harvested at the appropriate time thereafter, and protein, RNA, DNA and polyamines were extracted from them with 0.2 M-HClO₄ and determined quantitatively as described previously (Melvin & Keir, 1978a).

Determination of DNA synthesis

Cells growing in 2.24-litre roller bottles were dispensed into 50mm-diameter plastic dishes, each containing three 10mm-diameter glass coverslips, at a density of 0.3×10^6 cells per dish, and the cells were taken to 'resting' in medium containing 1% (v/v) serum, then 'stepped-up' as described above. After the appropriate time the medium was replaced with fresh medium containing 10% (v/v) serum and [6-³H]thymidine (2.0 μCi/ml). The radioactive medium was left in contact with the cells for 1 h at 37°C in a 5% CO₂ atmosphere; it was then removed, and the incorporation of [³H]thymidine into acid-precipitable material on each coverslip was determined (Craig *et al.*, 1975).

Assay of ornithine decarboxylase activity

Thin monolayers of BHK-21/C13 cells, growing in medium containing 10% (v/v) serum, were washed twice *in situ* with warm (37°C) medium containing 1% (v/v) serum and then were incubated for 4–5 days in 1% serum medium with one further change of

medium (after 48 h). The cells were 'stepped-up' by replacing the medium with fresh medium containing 1% (v/v) serum and cation, if required, followed 30 min later by addition of serum to a final concentration of 10% (v/v). At the appropriate time thereafter the cell sheet was washed with ice-cold 0.9% NaCl containing 20 mM-Hepes and 1 mM-dithiothreitol, pH 7.2. The cells were scraped off in the same buffer and were collected by centrifugation at 200g_{av.} for 10 min at 4°C. The cell pellet was resuspended in ice-cold 100 mM-Hepes containing 1 mM-dithiothreitol, pH 7.2, and was left for 10 min in an ice bath before being homogenized by 20 passes of a close-fitting pestle driven by a Tri-R Stir-R motor at 2500 rev./min. A sample of the homogenate was removed for determination of protein, and the remainder was centrifuged at 16000g_{av.} for 30 min at 4°C in an MSE High Speed 18 centrifuge. The supernatant was decanted and assayed immediately for ornithine decarboxylase activity by a modification (Howard *et al.*, 1974) of the method of Russell & Snyder (1968).

Results

Growth of BHK-21/C13 cells after addition of serum to serum-deprived cultures

Cells were incubated for 5 days in medium containing 1% (v/v) serum. Sterile MgCl₂, NaCl or KCl was added to the cultures to increase the concentration of cation in the medium by 15 mM, and 30 min later serum was added to a final concentration of 10% (v/v). For control cultures, the same amount of sterile 0.9% NaCl was added to the dishes 30 min before the serum. The results in Table 1 show that the growth of BHK-21/C13 cells was not inhibited by

Table 1. Growth of cells after re-addition of serum to 10% (v/v) to serum-starved cultures

Protein, RNA and DNA were extracted from cultures in 90mm-diameter dishes 48 h after addition of serum. Four dishes were assayed in triplicate for protein, RNA and DNA, and the results presented are the average content per dish. The incorporation of [³H]thymidine into DNA was measured 24 h after addition of serum, and the values shown represent the mean ± s.d. c.p.m. per coverslip, from six determinations.

Concn. of serum in medium (%, v/v)	Cation added	Protein (mg)	RNA (μg)	DNA (μg)	[³ H]Thymidine incorporated (c.p.m.)
1	None	0.25	27	10	861 ± 141
10	None	0.78	96	25	8840 ± 1365
10	Mg ²⁺	0.74	97	22	9103 ± 571
10	Na ⁺	0.82	108	21	7426 ± 835
10	K ⁺	1.13	138	24	7681 ± 555

increasing the concentration of Mg^{2+} , Na^{+} or K^{+} ions in the medium by 15 mM before addition of the serum.

Synthesis of polyamines after serum 'step-up' and the effects of exogenous cations

Resting serum-deprived cultures of BHK-21/C13 cells were stimulated to grow by re-addition of serum to the medium to a final concentration of 10% (v/v), both in the absence and presence of additional exogenous $MgCl_2$, $NaCl$ or KCl , giving an increased concentration of cation in the medium of 15 mM. The accumulation of spermidine was inhibited by this extra amount of exogenous Mg^{2+} ions, and the molar ratio of spermidine/spermine was lower in cells stimulated to grow in medium containing an additional 15 mM concentration of Mg^{2+} ions compared with other cultures (Table 2).

Effect of cations on induction of ornithine decarboxylase activity

The activity of ornithine decarboxylase in serum-starved cultures of BHK-21/C13 cells is very low, but within 4–5 h of re-addition of serum there was a marked increase in the activity of this enzyme (Howard *et al.*, 1974). Addition of $MgCl_2$ to the culture medium (to give an increase in Mg^{2+} ion concentration of 30 mM) 30 min before addition of the serum severely inhibited the increase in ornithine decarboxylase activity, and this inhibition was maintained for at least 24 h (Table 3). However, the increase in ornithine decarboxylase activity was not completely prevented by the addition of a further 30 mM- $MgCl_2$ to the culture medium (Chen *et al.*, 1976). $NaCl$ or KCl , added to the culture medium to give an increase in cation concentration of 30 mM, did not inhibit the induction of ornithine decarboxylase activity in BHK-21/C13 cells, in contrast with results obtained from various other cell systems (Chen *et al.*, 1976).

The inhibition of ornithine decarboxylase activity observed when an additional 30 mM- Mg^{2+} was

Table 3. *Effect of exogenous cations on induction of ornithine decarboxylase activity*

The results shown are from three different experiments, *a*, *b* and *c*. In experiment *a* the cells were in 90 mm-diameter plastic dishes, and in experiments *b* and *c* they were in 2.24-litre roller bottles. The values given, which represent the means of triplicate assays, are expressed as a percentage of the enzyme activity in serum-stimulated cultures (100%). These 100% values were: *a*, 0.4; *b*, 0.24; *c*, 0.3 pmol of CO_2 released/h per μg of protein.

Concn. of serum in medium (% v/v)	Cation added	Ornithine decarboxylase activity (%)		
		<i>a</i> (4 h)	<i>b</i> (16 h)	<i>c</i> (24 h)
1	None	30	27	—
10	None	100	100	100
10	Mg^{2+}	18	26	20
10	Na^{+}	115	—	—
10	K^{+}	93	—	—

present in the culture medium before addition of serum was not due to inhibition of enzyme activity *in vitro* caused by excess Mg^{2+} ions in the assay mixture. The increase in Mg^{2+} concentration in the assay that would occur with extracts of cells incubated with additional 30 mM- $MgCl_2$ would be approx. 2 mM, assuming complete equilibration of intracellular and extracellular Mg^{2+} ions, and Fig. 1 illustrates that addition of this amount of $MgCl_2$ directly to an ornithine decarboxylase assay had little effect on enzyme activity.

Effect of methylglyoxal bis(guanylhydrazone) on cell growth, and the competing effects of Mg^{2+} ions and spermidine

Methylglyoxal bis(guanylhydrazone) added to the culture medium (to 20 μM) of resting serum-starved BHK-21/C13 cells 30 min before re-addition of serum considerably inhibited stimulation of cell growth

Table 2. *Effect of serum 'step-up' on cellular polyamine accumulation*

Polyamines were extracted from cultures in 90 mm-diameter dishes 48 h after addition of serum. Two dishes were assayed in triplicate and the values given represent the average content per dish. All values obtained varied by less than 10% from the average values.

Concn. of serum in medium (% v/v)	Cation added	Polyamine content (nmol)			Spermidine/spermine
		Spermine	Spermidine	Putrescine	
1	None	1.4	0.8	≤ 0.1	0.55
10	None	5.5	5.1	0.7	0.97
10	Mg^{2+}	6.4	3.7	0.6	0.58
10	Na^{+}	6.4	4.8	0.7	0.75
10	K^{+}	7.1	5.3	0.8	0.75

(Table 4). This inhibition was prevented by the addition of spermidine (to 100 μ M) together with the drug. Addition of $MgCl_2$, to 30 mM, together with the drug only partly overcame the inhibition of cell growth.

The effects of spermidine and Mg^{2+} ions in preventing the inhibitory action of methylglyoxal bis-(guanyldrazone) on the growth of BHK-21/C13 cells were not due to direct interactions between the drug and the cations, leading to a decrease in the amount of drug available to enter the cells. Spermidine and $MgCl_2$ at various concentrations had no effect on the absorbance spectrum of methylglyoxal bis(guanyldrazone) *in vitro* ($\lambda_{max.} = 283$ nm).

Fig. 1. Effect of $MgCl_2$ on the activity of ornithine decarboxylase *in vitro*

Ornithine decarboxylase was extracted from BHK-21/C13 cells growing exponentially in 2.24-litre roller bottles; 104 μ g of protein was used for each determination. The results represent the means of triplicate assays, and the vertical bars show the highest and lowest individual estimates. The values are shown as percentages of the activity measured in the absence of $MgCl_2$. This had a value of 3.5 pmol of CO_2 /h per μ g of protein, and represented 20.5×10^3 c.p.m. per assay.

Discussion

The effects of addition of excess exogenous cations to serum-starved cultures of BHK-21/C13 cells before addition of serum have been investigated. Of the three cations tested, Mg^{2+} , Na^+ and K^+ , only Mg^{2+} ions had an inhibitory effect on the increase in ornithine decarboxylase activity observed 4–5 h after re-addition of serum, and this inhibition was maintained for at least 24 h. Inhibition of ornithine decarboxylase activity in the presence of an increased concentration of Mg^{2+} ions in the medium was not complete, so that the syntheses of spermidine and spermine increased in those cells that had been incubated in medium containing additional Mg^{2+} ions before addition of serum, compared with 'resting' cells. However, accumulation of cellular spermidine was less in cells stimulated by serum in the presence of added Mg^{2+} ions compared with those that had received no additional cation, and this was reflected in the lower ratio of spermidine/spermine in these cells. The presence of increased amounts of Mg^{2+} ions in the medium did not inhibit the increase in cell growth that follows re-addition of serum to serum-deprived cultures; this suggests that Mg^{2+} ions may be able to replace polyamines in certain cellular reactions, particularly when the intracellular concentration of spermidine is decreased. Studies with plant tissues have suggested that plant polyamines and physiological cations may be interchangeable (Sinclair, 1967), and certainly in many biochemical reactions studied *in vitro* there occurs an overlap between the effects of the polyamines and of other cations, especially K^+ and Mg^{2+} (Tabor & Tabor, 1976; Cohen & Zalik, 1978).

In various cell systems studied *in vivo*, changes in cell growth rate correlate with changes in the intra-

Table 4. Effect of Mg^{2+} ions and spermidine on the inhibition of cell growth by methylglyoxal bis(guanyldrazone)

Cell cultures were in medium containing 1% serum for 4 days before 'step-up'. Protein, RNA and DNA were extracted from cultures 48 h after addition of serum. Four dishes were assayed in triplicate, and the values given are the average contents per dish. The incorporation of [3H]thymidine into DNA was measured 22 h after addition of serum to the cultures, and the values given represent means $\pm$ s.d. c.p.m. per coverslip, for the numbers of determinations in parentheses. The concentrations of cations used were: methylglyoxal bis(guanyldrazone) (Mgbgh), 20 μ M; spermidine, 100 μ M; $MgCl_2$, 30 mM.

Concn. of serum in medium (% v/v)	Cation added	Protein (mg)	RNA (μ g)	DNA (μ g)	[3H]Thymidine incorporated (c.p.m.)
1 (Resting cells)	None	—	—	—	725 $\pm$ 154 (6)
1	None	—	—	—	1720 $\pm$ 360 (6)
10	None	1.01	108	26	10105 $\pm$ 2300 (8)
10	Mg^{2+}	1.04	109	25	9253 $\pm$ 2168 (8)
10	Mgbgh	0.60	61	9	3150 $\pm$ 594 (9)
10	Mgbgh + Mg^{2+}	0.70	78	15	9410 $\pm$ 1460 (8)
10	Mgbgh + spermidine	0.98	104	19	11340 $\pm$ 1718 (9)
10	Spermidine	0.90	98	—	10036 $\pm$ 893 (5)

cellular concentrations of polyamines. Methylglyoxal bis(guanyldihydrazone), which inhibits the syntheses of spermidine and spermine, inhibits the growth of mammalian cells in culture (Fillingame *et al.*, 1975; Melvin & Keir, 1978b; Rupniak & Paul, 1978). This inhibition of cell growth can be overcome in some cell systems by addition of spermidine to cultures treated with methylglyoxal bis(guanyldihydrazone) (Rupniak & Paul, 1978). The addition of Mg^{2+} ions to cultures of BHK-21/C13 cells together with methylglyoxal bis(guanyldihydrazone) partially overcame the inhibitory effects of the drug on cell growth, but Mg^{2+} ions were not as effective as spermidine in this respect (Table 4). However, Mg^{2+} ions were particularly effective in overcoming the inhibition of DNA synthesis that occurs in cells incubated in the presence of methylglyoxal bis(guanyldihydrazone). This suggests that Mg^{2+} ions can be substituted quite successfully for spermidine and spermine in the process of DNA synthesis in BHK-21/C13 cells, whereas the requirement for polyamines as cations in other cell processes, such as the syntheses of RNA and protein, is more specific and cannot be satisfied by Mg^{2+} ions.

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