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Rb^+ Influxes Differentiate between Growth Arrest of Cells by Different Agents

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Summary. The effect of cell cycle on Rb^+ (K^+) fluxes was studied in NIH 3T3 mouse fibroblasts. Serum starvation or isoleucine deprivation resulted in cell arrest at an early G_1/G_0 phase, accompanied by a marked decrease in both ouabain-sensitive and ouabain-resistant Rb^+ influx. On the other hand, cells arrested at late G_1/G_0 phase by hydroxyurea treatment have high ouabain-sensitive and ouabain-resistant Rb^+ influx. Butyric acid treatment resulted in cell arrest at an early G_1/G_0 phase, but in contrast to serum or isoleucine starvation did not decrease Rb^+ influxes. It is thus shown that quiescent cells may have Rb^+ influx rates as high as that of logarithmically growing cells. The results are consistent with the hypothesis that an increased ion permeability of the cell is initiated at a critical stage in G_1/G_0 phase, and that butyric acid may arrest the cell beyond that stage.

Key Words ouabain-resistant · ouabain-sensitive · Rb^+ influx

Introduction

Quiescent cells arrested at the G_1/G_0 phase of the cell cycle have a very low Na^+/K^+ pump activity. The addition of serum to these quiescent cells was accompanied by a rapid stimulation of the Na^+/K^+ pump (Rozengurt & Heppel, 1975; Smith, 1977; Tupper, Zorgniotti & Mills, 1977; Panet, Fromer & Atlan, 1982). This activation appears to be a result of increased Na^+ entry into the released cells (Smith & Rozengurt, 1978*a*, *b*; Knock & Leffert, 1979). It has been proposed that early changes in the ion fluxes (K^+ and Na^+) signal the initiation of cell proliferation (Rozengurt, 1980). The total K^+ and Na^+ fluxes through cell membrane have been shown to be composed of three components: (a) Na^+/K^+ pump; (b) ouabain-resistant fluxes; and (c) residual flux which have characteristics of diffusion. (Tupper et al., 1977; Bakker-Grunwald, 1978; Geck et al. 1980; Bakker-Grunwald, Andrew & Neville, 1980). The ouabain-resistant K^+ (or Rb^+) influx has several distinguishable properties: self exchange, dependence upon chloride ion, and sensitivity to diuretic drugs such as furosemide and ethacrinic acid (Bakker-Grunwald, 1978; Geck et al., 1980). This influx is believed to participate in cell volume regulation (Geck et al., 1980; Lauf & Valet, 1980). We have previously described a method to differentiate among the three K^+ fluxes (Panet & Atlan, 1980). This method was applied to measure the different K^+ fluxes in NIH 3T3 mouse fibroblasts which were synchronized by serum starvation (Panet et al., 1982). The addition of serum to quiescent NIH 3T3 mouse cells resulted in a 10 to 20-fold increase in Rb^+ influx which was resistant to ouabain and in three- to fourfold activation of the ouabain-sensitive influx. The ouabain-resistant Rb^+ influx in the released cells was not affected by amiloride and monensin, and was sensitive to ethacrinic acid in addition to being dependent on the presence of chloride ions (Panet et al., 1982).

In this communication we extended these studies to cells synchronized by different agents at different sites of the cell cycle. This was done in order to see whether the stimulation of Rb^+ (K^+) influxes is a general phenomenon for released cells, and whether it is an essential event which signals the initiation of cell proliferation.

Materials and Methods

$^{86}Rb^+$ was purchased from the Radiochemical Centre Amersham. ^3H-Thymidine was purchased from the Nuclear Research Centre, Beer-Sheva, Israel.

Cell synchronization: NIH 3T3 mouse cells (Jainchill, Aaronson & Todaro, 1969) (200,000) were plated in 35-mm dishes (Nunc) with RPMI 1640 medium containing 10% calf serum.

(a) Serum starved cultures were prepared as described before (Panet et al., 1982); (b) Isoleucine-depleted cultures were obtained by replacing the growth medium after 48 hr by minimal Eagle's medium (MEM) deficient of isoleucine, containing 10% dialyzed fetal calf serum for 48 hr; (c) Butyric acid treated cells: the growth medium was replaced by RPMI medium containing 10% calf serum and 5 mM butyric acid for 48 hr; (d) Hydroxyurea-treated cultures: RPMI medium containing 10% calf serum and 2 mM hydroxyurea was added to serum-depleted cultures (*see* a) for 24 hr.

Ouabain-sensitive (OS) and ouabain-resistant (OR) Rb^+ influxes, and Rb^+ influx by diffusion were measured as reported before (Panet & Atlan, 1980). In brief, Rb^+ influx in the presence of ouabain was subtracted from total Rb^+ influx and taken as OS Rb^+ influx. Rb^+ influx in the presence of ouabain and isotonic KCl has the characteristics of a diffusion flux (Panet & Atlan, 1980) and was taken as influx through diffusion. Rb^+ influx by diffusion was subtracted from total ouabain-resistant influx to give the ouabain-resistant carrier-mediated Rb^+ influx (OR). The assay was linear up to 20 min and was normally conducted for 5 min. Rb^+ influx rates presented throughout this work are average of triplicate cultures. DNA synthesis was measured by adding ^{3}H Thymidine (2 µCi) to the growth medium. After 30 min at 37 °C the medium was removed, cultures were washed with PBS, incubated with cold 5% TCA (2 ml), and washed three times with cold TCA (5%). The cells were lyzed and counted as described before (Panet et al., 1982).

Results

Ouabain-Sensitive and Resistant Rb^+ Influxes in Arrested and Released Cultures

Rb^+ influx rates, namely OS, OR and Rb^+ influx by diffusion were compared in NIH 3T3 cells arrested by the following procedures: (a) serum starvation; (b) isoleucine deprivation; (c) treatment with butyric acid; and (d) treatment with hydroxyurea (Table 1). Cultures arrested by serum and isoleucine starvation have relatively low Rb^+ influx rates. In contrast, cells arrested by butyric acid and hydroxyurea treatments have high OS and OR Rb^+ influx rates, with no apparent change in Rb^+ influx by diffusion (Table 1).

Serum starvation, isoleucine deprivation, and butyric acid treatment have been reported to arrest cells at the G_1/G_0 phase of the cell cycle (Pardee, 1974; D'Anna et al., 1980; Kruh, 1982); hydroxyurea, on the other hand, is known to block the cell cycle at the early *S*-phase (Pardee, 1974).

In a previous work, we have shown that release of quiescent NIH 3T3 cells from the G_1/G_0 phase by the addition of serum resulted in a marked increase of both OS and OR Rb^+ influx rates (Panet et al., 1982). In this work Rb^+ influx rates after release of cultures from arrest by isoleucine deprivation, serum starvation, butyric acid and hydroxyurea treatments were compared (Fig. 1). Release of cultures arrested by isoleucine deprivation resulted in a marked increase of Rb^+ influxes OS and OR by three- and five-fold, respectively (Fig. 1A). The OS Rb^+ influxes stimulated by the addition of isoleucine remained high for 4 hr (Fig. 1*A*). The OR, however, remained high up to 1 hr and then declined. This is in contrast to the

Table 1. Effect of different blocking conditions on Rb^+ influxes[a]

Method of arrest	Rb^+ influx		
	OS	OR	Passive diffusion
	(pmol/min/µg protein)		
Growing cells	17.0±0.8	13.6±1.4	2.2±0.3
Serum starvation	9.4±0.9	3.2±1.3	1.8±0.4
Isoleucine deprivation	5.1±0.9	4.3±1.4	2.2±0.5
Butyric acid treatment	22.7±0.5	20.3±1.5	2.1±0.4
Hydroxyurea treatment	19.3±0.9	11.5±0.1	1.8±0.1

[a] Cells were arrested and Rb^+ influx rates were assayed as described in materials and methods. Results presented in this table are average of five independent experiments and presented as mean±SD.

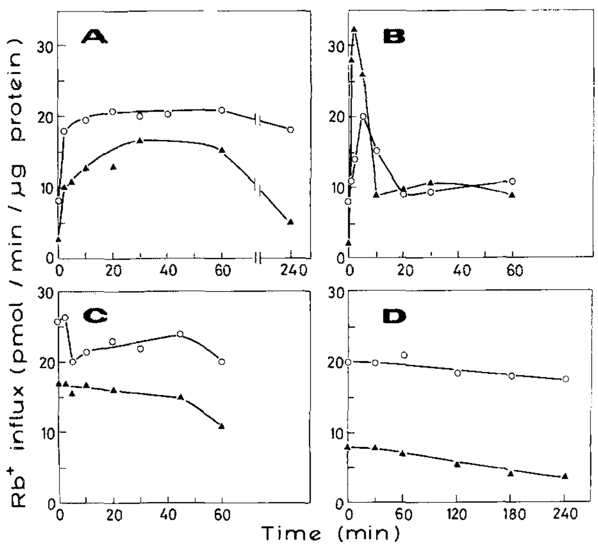

Fig. 1. Kinetics of OS and OR Rb^+ influx activation following release of arrested cells by different agents. Quiescent cells were arrested by (*A*) isoleucine deprivation; (*B*) serum starvation; (*C*) butyric acid treatment; and (*D*) hydroxyurea treatment, as described in Materials and Methods. The quiescent cells were stimulated by washing the plates with PBS and then adding complete medium containing 10% calf serum. Rb^+ influx was assayed as described in Materials and Methods: (o) ouabain-sensitive Rb^+ influx; (▴) ouabain-resistant Rb^+ influx

fast decline of the serum-stimulated OS and OR Rb^+ influxes (Fig. 1*B*) (Rozengurt & Heppel, 1975; Panet et al., 1982).

The high OS and OR Rb^+ influx rates found in the butyric acid and hydroxyurea arrested cells, have not been greatly affected after release (Fig. 1*C, D*). It appeared therefore that cells arrested by serum starvation and isoleucine deprivation were similar in their low Rb^+ influxes and in the fast stimulation following release. On the other hand, Rb^+ influxes in cells arrested by bu-

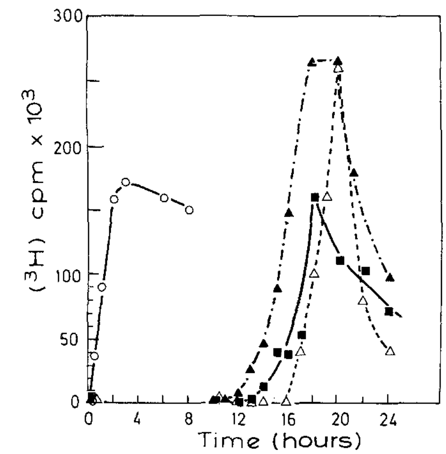

Fig. 2. DNA synthesis kinetics after releasing arrested cells. Cells arrested by (△) isoleucine deprivation; (▲) serum starvation; (■) butyric acid treatment; (o) hydroxyurea treatment, as described in Materials and Methods. The quiescent cells were washed with PBS; complete medium containing 10% calf serum was added, and DNA synthesis was measured as described in Materials and Methods

tyric acid and hydroxyurea treatment were high and independent of cell release (Table 1, Fig. 1).

Does Butyric Acid Treatment and Isoleucine Deprivation Arrest the Cells at the Same Site?

Since butyric acid-arrested cells had high Rb^+ influxes as compared to cultures arrested by serum and isoleucine starvation, it was of interest to determine whether butyric acid arrested the cells at the same phase. In order to compare the precise site into which the blocking conditions arrested the cells, the progression into *S* phase (lag of DNA synthesis) was measured after the release. Release from hydroxyurea treatment resulted in an immediate DNA synthesis (Fig. 2), in agreement with reports showing that this compound arrested cells in the early *S* phase (Pardee, 1974). On the other hand, DNA synthesis in the other three released cultures peaked at 18–20 hours (Fig. 2). Some differences were observed as to the width of the *S* phase (Fig. 2); this could be attributed to the degree of synchronization by the different arresting procedures. These results indicated that arrest by serum starvation, isoleucine deprivation and butyric acid treatment blocked the cells at a similar site in G_1/G_0 phase. Nevertheless, determination of arrest sites in the G_1/G_0 phase by the thymidine incorporation kinetics is not precise enough. There is some controversy over whether isoleucine and serum deprived cells are blocked at the same site or not (Pardee, 1974; Martin & Stein, 1976; Burstin, Meiss & Basilico, 1974; Kohn, 1975). Our results are compatible with the conclusion that the two quiescent cell populations were in the same state (Table 1, Figs. 1 and 2).

An important question arose whether the high Rb^+ influx rates of butyric acid-treated cultures were the result of cell arrest at a different site from that of serum or isoleucine starvation. If, for example, butyric acid arrested the cultures ten minutes or more beyond the site of arrest by serum and isoleucine starvation, the OS and OR Rb^+ influxes would be expected to be high (Fig. 1). To determine the exact position of cell arrest by butyric acid, we used the two sequential blocking methods described by Pardee (1974). This test determines whether quiescent cells blocked by two different procedures are at the same site. By imposing one block and then releasing the cultures in the presence of another kind of block, one could determine the relative sites of the two blocks. If the block applied second arrested the cells after or at the same point as the one applied earlier, the cells would not proceed to *S* phase after release from the first block. If however, the second block arrested the cells at an earlier phase than the first block, the cell should be able to proceed to *S* phase after release from the first block.

Table 2 summarizes two double block experiments using butyric acid treatment and isoleucine deprivation. In the first experiment (Table 2A) butyric acid was applied for 48 hr and replaced by the second block (minus isoleucine) for an additional 24 hr. It was evident that the cells did not proceed to *S* phase by applying isoleucine starvation after butyric acid treatment. Experiments using butyric acid as the second block were more complex, since 36–48 hr were required to arrest cells by butyric acid (Kruh, 1982). To overcome this complication, butyric acid was added for 24 hr before the release from isoleucine deprivation. Cultures released by this procedure (Table 2*B*) synthesized only little DNA (7.4%) as compared to control cultures. The residual DNA synthesis in these cultures could be attributed to the slow effect of butyric acid. It should be noted that values below 20% of thymidine incorporation in double block experiments indicated that the two treatments blocked at the same site (Pardee, 1974). The above experiments suggest that butyric acid and isoleucine starvation block the cells at the same site or very close in the G_1/G_0 phase.

Mechanism of Butyric Acid Effect on Rb^+ Influxes

There are two possible explanations for the high OS and OR Rb^+ influxes found in butyric acid-

Table 2. DNA synthesis in cultures synchronyzed by double block procedures[a]

Method of arrest	First change	Second change	Thymidine incorporation % of control
A			
1. Butyric acid	–	Complete medium	100
2. Butyric acid	Minus isoleucine	–	1.28
3. Butyric acid	–	–	0.09
B			
1. Isoleucine deprivation	Minus isoleucine plus butyric acid	Complete medium	100
2. Isoleucine deprivation	Minus isoleucine plus butyric acid	Complete medium plus butyric acid	7.4
3. Isoleucine deprivation	–	–	0.25

[a] *A*: Cells were treated with butyric acid as described in Materials and Methods for 48 hr (*A*-3). Arrested cultures were washed with PBS, fed with MEM medium deficient of isoleucine (*A*-2), or complete medium as control (*A*-1), and after 24 hr, thymidine incorporation was measured as described in Materials and Methods.

B: Cells were arrested by isoleucine starvation as described in Materials and Methods, and 5 mM butyric acid was added to the same medium for another 24 hr (*B*-1, *B*-2). The medium was changed by complete medium (*B*-1) or by complete medium containing butyric acid (*B*-2) for another 24 hr, and thymidine incorporation was measured. Thymidine incorporation in control cultures released by the addition of complete medium (100%) was 196,500 cpm in butyric acid-treated cells (*A*-1) and 76,000 cpm in isoleucine deprived cells (*B*-1).

Table 3. Effect of butyric acid on Rb^+ influxes in isoleucine-starved cultures[a]

Method of arrest	Addition after 24 hr	Addition after 48 hr	Rb influx	
			OS	OR
			(pmol/min/μg protein)	
a. Minus isoleucine	Minus isolucine	–	7.1 ± 0.4	3.5 ± 0.2
b. Minus isoleucine	Minus isoleucine plus butyric acid	–	6.0 ± 0.1	4.0 ± 0.2
c. Minus isoleucine	Minus isoleucine plus butyric acid	Plus isoleucine plus butyric acid	22.9 ± 1.0	13.8 ± 0.1
d. Butyric acid	Plus butyric acid	–	16.8 ± 0.4	11.0 ± 0.3

[a] Cells were arrested by isoleucine starvation for 24 hr as described in Materials and Methods. Addition after 24 hr: the medium was replaced by a medium deficient of isoleucine with (*b*, *c*) and without (*a*) butyric acid for another 24 hr. Addition after 48 hr: the isoleucine deficient medium which contained butyric acid was replaced by a complete medium plus 5 mM butyric acid for another 24 hr (*c*). Cultures arrested by 5 mM butyric acid (*d*).

The results presented in this table are mean of triplicate cultures ± SD.

treated cells: (i) butyric acid directly affects the cell membrane; or (ii) the high Rb^+ influxes could be a consequence of the site of arrest by butyric acid. If butyric acid has a direct effect on the Rb^+ influxes it might stimulate the low Rb^+ influxes in isoleucine-deprived cells. We treated isoleucine-deprived cells with butyric acid for 24 hr; the OS and OR Rb^+ influxes remained low in the absence of isoleucine (Table 3*b*). In addition, when butyric acid was added directly to the assay mixture or to the complete medium during release of isoleucine deprived cells, it did not affect the OS and OR Rb^+ influxes (not shown here). This result suggested that butyric acid did not activate Rb^+ influxes through a direct effect on the cell membrane. To test the second possibility we released isoleucine-starved cultures in the presence of butyric acid for 24 hr (Table 3*c*). These cultures although unable to proceed to *S* phase (Table 2*B*2) have high OS and OR Rb^+ influxes. The high Rb^+ influx rates could not be detected unless the cells were released from isoleucine starvation (Table 3*b*).

Thus the effect of butyric acid treatment on Rb^+ influxes appeared to be related to cell cycle.

Discussion

In this communication we described the effects of cell cycle on three different Rb^+ (as analog of K^+) influxes. Both serum-starved and isoleucine-deprived cells demonstrated low OS and OR Rb^+ influxes as compared to growing cells. In contrast, cells arrested by butyric acid treatment have high OS and OR Rb^+ influxes (Table 1). This finding was unexpected since it has been reported before that butyric acid arrests cells at G_1 phase (D'Anna et al., 1980). In fact, with thymidine incorporation and double block experiments (Pardee, 1974) we confirmed this finding (Fig. 2, Table 2). Following release of cells from isoleucine deprivation and butyric acid treatment, DNA synthesis peaked at 18–20 hr, and a cell arrested by one block could not be released in the presence of the other block. Moreover, our results indicate that butyric acid does not have a direct effect on the cell membrane (Table 3).

There are several possible explanations for these observations:

(a) Butyric acid treatment, isoleucine deprivation, and serum starvation arrested cells at the same site of the cell cycle, as suggested by the double block experiments (Table 2). If this is the case, then the high Rb^+ influxes in butyric acid-treated cells is not the consequence of the site of cell arrest, but rather a result of the specific treatment. The finding that butyric acid added for 24 hr could not activate the low OS and OR Rb^+ influxes in isoleucine-deprived cells (Table 3*b*) may rule out this possibility.

(b) It is possible, however, that butyric acid arrested the cells close but beyond the site of arrest caused by isoleucine starvation. At this site of the cell cycle, the Rb^+ influxes would be expected to be high (Fig. 1). The finding that releasing the isoleucine-deprived cells in the presence of butyric acid stimulated OS and OR Rb^+ influxes (Table 3*c*) may support this possibility; the stimulation of Rb^+ influxes in the arrested cells by butyric acid could not be detected unless isoleucine was added (Table 3). The addition of isoleucine in the presence of butyric acid could not release the cells to enter *S* phase (Table 2*B*); nonetheless, it did stimulate the Rb^+ influxes (Table 3*c*). The above result suggested that butyric acid arrested the cell between isoleucine site and *S* phase. If indeed, butyric acid arrested the cells at G_1 phase beyond the site of arrest by isoleucine, or serum, then the activities of Rb^+ influxes could be used as a sensitive marker for this site.

The Differentiation between OR and OS Rb^+ Influxes in Quiescent and Released Cells

In recent years there has been renewed interest in ouabain-resistant diuretic-sensitive K^+ and Na^+ fluxes, shown to exist in many cells (Tupper et al., 1977, Bakker-Grunwald, 1978; Geck et al., 1980; Bakker-Grunwald et al., 1980). The exact role and mechanism of this transport system is not completely understood.

The relationship between OS and OR Na^+ and K^+ fluxes may regulate the cell ionic composition and volume (Geck et al., 1980). The ratio between OR and OS Rb^+ influxes in isoleucine and serum-deprived cells was found to be 1:2–2.5 (Table 1). Similar ratio was found in serum-starved cells by others (Tupper et al., 1977). Both OR and OS Rb^+ influxes were stimulated following the addition of serum to quiescent cells (Tupper et al., 1977; Panet et al., 1982). In this communication we find that the addition of isoleucine to isoleucine-deprived cells results in a stimulation of the OS and OR Rb^+ influxes similar to the stimulation of these fluxes upon the addition of serum to serum-starved cells (Fig. 1). We interpreted these results as an indication that serum-starved and isoleucine-deprived cells are at the same stage of the cell cycle (Pardee, 1974). The finding that the release of arrested cells results in the stimulation of both OR and OS Rb^+ influxes suggests that it may be a general event in the early G_1/G_0 phase. It is of interest that in both isoleucine and serum-starved cells the OR to OS Rb^+ influx ratio increases similarly from 1:2.5 (OR to OS) to 1:1. In other words, the activation degree of OR Rb^+ influx is higher than that of OS influx (Panet et al., 1982; Fig. 1; Table 3). The OR Rb^+ influx was found to be sensitive to ethacrinic acid and chloride ion dependent (Panet et al., 1982), which rule out the possibility that it is due to incomplete inhibition of the Na^+/K^+ pump.

In summary, our experiments may suggest that release of arrested cells is accompanied by stimulation of the K^+ fluxes, only at specific sites of the G_1/G_0 phase, probably at the very early phase.

It is interesting to note that the activity of Na^+/K^+ pump is not dependent upon the presence of growth factors, since isoleucine-deprived cells exhibit low Na^+/K^+ pump in the presence of serum and could be stimulated by adding isoleucine only. It is conceivable that the modulation of the Na^+/K^+ pump is cell-cycle dependent.

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