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Differentiation between Serum Stimulation of Ouabain-Resistant and Sensitive Rb Influx in Quiescent NIH 3T3 Cells

Rivka Panet, Ilana Fromer, and Henri Atlan
Department of Medical Biophysics and Nuclear Medicine, Hadassah University Hospital, Jerusalem, Israel

Summary. The addition of serum to quiescent NIH 3T3 mouse cell cultures resulted in a 10- to 20-fold increase of Rb influx which was resistant to ouabain, and only a three- to fourfold activation of ouabain-sensitive Rb influx. Stimulation of the ouabain-resistant Rb influx following serum addition reached its maximum within 2 min. The stimulation of ouabain-resistant Rb influx was a result of V_m increase while the K_m for Rb was unchanged. Ouabain-resistant Rb influx, after serum addition, was resistant to amiloride and sensitive to ethacrinic acid. Replacing chloride in the medium by NO_3^-, CO_3^- and CH_3COO^- resulted in a drastic decrease in the ouabain-resistant Rb influx. It appeared, therefore, that the ouabain-resistant Rb influx in NIH 3T3 cells was Cl^--dependent.

Key words ouabain-resistant · Rb-influx · serum-stimulation

Introduction

Depletion of growth factors from cells in a culture resulted in the arrest at the G_1/G_0 phase of the cell cycle (Todaro and Green, 1963; Holley, 1975). The addition of serum or purified growth factors induced an array of events in quiescent cultures which preceded the onset of DNA synthesis (*S*-phase) and cell division (*M*-phase) (Tupper, Zorgniotti & Mills, 1977; Pardee, Dubrow, Hamlin & Kletzien, 1978). One of the earliest changes observed following serum addition was an increase in the activity of the Na/K-pump (Rozengurt & Heppel, 1975; Smith, 1977; Tupper et al., 1977). It has been proposed that the effect of growth-promoting factors on the Na/K-pump was mediated by an increased transport of Na into the cells, through activation of specific channels, which result in an increased supply of Na to the Na/K-pump (Smith & Rozengurt, 1978*a, b*).

In a previous publication we described a method to differentiate between the different K fluxes: (1) Ouabain-sensitive influx; (2) ouabain-resistant influx; and (3) K (Rb) influx by plain diffusion (Panet & Atlan, 1980). In this communication we have applied this assay to study Rb influxes following the addition of serum to quiescent cells.

Materials and Methods

DNDS was kindly given by Dr. Cabantchik of the Hebrew University, Jerusalem, Israel. Ethacrinic acid was obtained from Merck, Sharp and Dohme. ^{86}Rb was purchased from New England Nuclear. RPMI medium was obtained from Gibco, Grand Island, New York.

Cell and Culturing Conditions

NIH 3T3 mouse cells (Jainchill, Aaronson & Todaro, 1969) were maintained in RPMI 1640 medium containing 10% calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin, in humidified atmosphere of 10% CO_2, 90% air at 37 °C. Quiescent cultures were obtained by plating cells (200,000) in 35-mm dishes (Nunc) with medium containing 10% calf serum. After 2 to 3 days the medium was replaced with medium containing 0.5% calf serum and one to two days later cells arrested at G_o phase were tested by ^3H thymidine incorporation. Thymidine incorporation started to increase at 11 hr and peaked at 17 hr (mid *S*-phase). In this work we used only cultures where DNA synthesis was less than 5% in comparison with similar cultures given fresh medium 24 hr earlier. Growing subconfluent cells were obtained by plating 100,000 cells which were used a day later.

Preparation of Cells for Rb Influx Measurement

(*i*) Serum-stimulated cultures: the medium was removed from the quiescent cultures (70–100 μg protein per 35-mm plate) and fresh medium (2 ml) containing 10% calf serum was added for 2 min at 37 °C. After 2 min (unless otherwise indicated), Rb influx was assayed as described below. (*ii*) Quiescent and growing cell cultures: the cells were washed with isotonic NaCl and Rb influx was assayed as described below.

Rb Influx Measurement

(a) Total Rb Influx. The reaction was begun by adding 2 ml assay mixture (in mM: 150 NaCl, 5 RbCl, 2 μCi ^{86}Rb, 2.5 Na-phosphate buffer, pH 7.0, 0.5 $CaCl_2$, 5 $MgCl_2$ and 10% calf serum). Incubations were carried out for 2 min (or longer where indicated) at 37 °C. Rb uptake was terminated by aspiration of the assay mixture and rapidly washing the cells four times with ice-cold solution containing 125 mM $MgCl_2$. The cells were lysed at 37 °C for 60 min in 0.6 ml solution containing SDS (0.1%) and NaOH (0.1 N); radioactivity was counted in Toluene-Triton scintillation medium.

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(b) Ouabain-Resistant Rb Influx. The reaction was the same as for total Rb influx (*see* Section **a**) with the exception that 1.3 mM ouabain was added. From the ouabain dose response, we found that 0.5 mM ouabain was enough to complete suppression of Na/K-pump in the assay condition (Fig. 3). We, however, used 1.3 mM ouabain to ensure accurate measurement of the ouabain-resistant influx.

(c) Rb Influx by Diffusion in the Presence of Ouabain. The reaction mixture was the same as for total ouabain-resistant Rb influx (Section **b**) with the exception that NaCl was replaced by isotonic KCl solution. KCl was added in order to saturate the ouabain-resistant K^+ carrier. Rb influx in the presence of ouabain and KCl has the characteristics of diffusion flux (Panet and Atlan, 1980).

The calculation of ouabain-sensitive and resistant Rb influx was carried out as follows: (i) Ouabain-sensitive Rb influx was calculated by subtracting the radioactivity obtained for ouabain-resistant Rb influx (**b**) from the total Rb influx (**a**). (ii) Ouabain-resistant, carrier-mediated Rb influx was calculated by subtracting the radioactivity obtained from the Rb influx in KCl medium (Rb influx by diffusion **c**), from the total ouabain-resistant Rb influx (**b**).

Results

The results illustrated in Fig. 1 indicated that the addition of serum to quiescent NIH 3T3 fibroblasts markedly increased the rate of both ouabain-resistant and ouabain-sensitive Rb influx. The ouabain-resistant Rb influx was stimulated 10- to 20-fold and reached a maximum within 2 min. Later, this influx dropped to a level of about five-fold higher than the Rb influx of serum-depleted cultures. The transient activity of ouabain-resistant Rb influx could be observed only when the assay was short (2 min). In accordance with the results obtained by others (Rozengurt & Heppel, 1975), serum also stimulated ouabain-sensitive Rb influx in the cell by three- to fourfold. Ouabain-sensitive Rb influx increased within 1 min after serum addition, reached a maximum within 5 min and dropped slowly. The two Rb influxes stimulated by serum, namely ouabain-resistant and ouabain-sensitive, had similar maxima (20–32 pmol/min/μg protein).

The rapid stimulation of ouabain-resistant Rb influx was due to an increase in the V_m with no apparent change in the Michaelis constant (K_m 2.2 mM) (Fig. 2). V_m was found to be 4.5 pmol/min/μg protein in serum-depleted cultures and 32.3 pmol/min/μg protein after serum stimulation (Fig. 2). This type of kinetics has the characteristics of a carrier-mediated influx also found in other systems (Sachs, Kanuf & Dunham, 1975; Panet & Atlan, 1980).

The K_m of ouabain-sensitive Rb influx was found to be 1.2 mM (results not shown here), which was similar to results obtained by others (Rozengurt & Heppel, 1975). We used, therefore, 5 mM Rb in the assay which completely saturated the Na/K-pump and only 70% of the ouabain-resistant Rb influx (Fig. 2). High Rb or K concentrations reduce ouabain binding; in order to ensure complete inhibition of ouabain-sensitive Rb influx we did not increase Rb concentration above 5 mM.

Since accurate measurement of ouabain-resistant Rb influx depends upon the complete suppression of the Na/K-pump, the dose response for

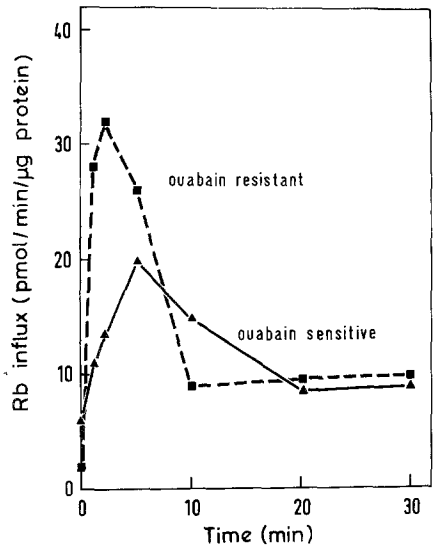

Fig. 1. Kinetics of ouabain-sensitive and resistant Rb influx activation after the addition of serum. Quiescent cultures were stimulated by adding medium containing 10% calf serum. Rb influxes were assayed as described in Materials and Methods. Zero-time Rb influxes were measured in the absence of serum in the assay mixture

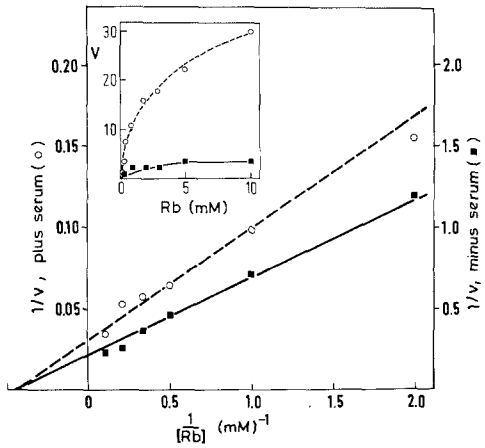

Fig. 2. Effect of Rb concentration on ouabain-resistant Rb influx. Ouabain-resistant Rb influx was assayed with quiescent cells as described in Materials and Methods, except that serum was omitted from the assay mixture. Serum-stimulated cultures were obtained and Rb influx was assayed as described in Materials and Methods. Rb influx rate was expressed in pmol/min/μg protein

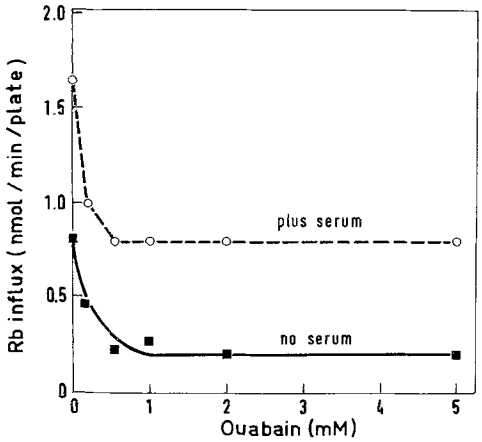

Fig. 3. Ouabain dose response for Rb influx. Quiescent and serum-stimulated cultures were obtained as described in Materials and Methods. Rb influx was assayed as a function of increased ouabain concentration. (■) quiescent cultures: serum was omitted from the assay mixture: (o) serum-stimulated cultures: medium (2 ml) containing 10% serum was added to the quiescent cultures for 5 min and Rb influx was assayed as described in Materials and Methods

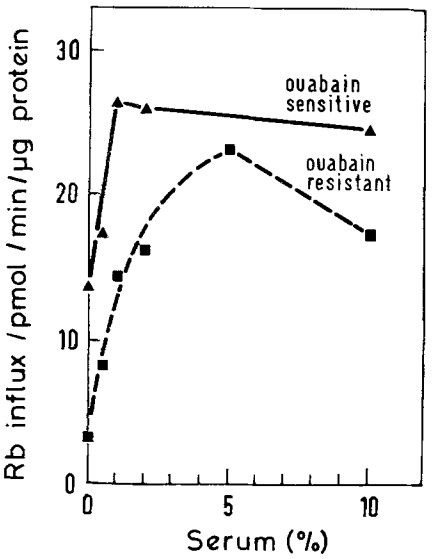

Fig. 4. Serum dose response of ouabain-resistant and sensitive Rb influxes. Medium containing increasing serum concentrations was added to quiescent cells. After 2 min at 37 °C the medium was removed and Rb influxes were assayed as described in Materials and Methods, with 10% serum

ouabain had to be studied. Results shown in Fig. 3 indicated that 0.5 mM ouabain maximally inhibited serum-depleted and serum-stimulated cell Na/K-pump. We used, therefore, 1.3 mM ouabain in the assay. To analyze the dose response of the two Rb influxes for serum, increasing concentrations of serum were added to quiescent cells. While 5% serum was needed for maximal activation of ouabain-resistant influx, 1% serum was sufficient for the ouabain-sensitive influx (Fig. 4).

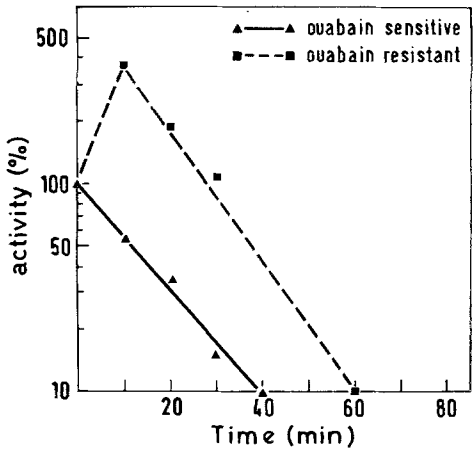

Fig. 5. Thermolability of ouabain-sensitive and -resistant Rb influxes. Growing cultures were incubated at 50 °C and at time intervals. Rb influxes were assayed at 37 °C for 5 min as described in Materials and Methods. The Rb influxes at zero time (100%) were 3.61 and 1.33 nmol/min/plate for ouabain-sensitive and ouabain-resistant, respectively

Table 1. The Effect of ethacrinic acid on ouabain-sensitive and ouabain-resistant Rb influx[a]

Ethacrinic acid concentration (mM)	Rb influx (%)	
	Ouabain-sensitive	Ouabain-resistant
0	100	100
0.1	110	93
0.5	125	72
1	102	26
2	71	13

[a] Serum-stimulated cell cultures were obtained and Rb influx activities were assayed as described in Materials and Methods in the presence of different ethacrinic acid concentrations. Control activities (100%) were 14.6 and 25.0 pmol/min/µg protein for ouabain-sensitive and ouabain-resistant influxes, respectively.

To further differentiate between the two Rb influxes, their thermolability was compared (Fig. 5). Ouabain-resistant influx was 3.5-fold activated after short incubation at 50 °C and then slowly inactivated with a half-life of 33 min. The ouabain-sensitive Rb influx was more thermolable with a half-life of 12 min.

The two Rb influxes could also be separated with the use of specific inhibitors. Thus, ouabain-resistant, in contrast to ouabain-sensitive influx was inhibited by ethacrinic acid (Table 1). Amiloride, which is known to decrease Na permeability in a variety of tissues (Bentely, 1968; Johnson, Epel & Paul, 1976; Sudou & Hoshi, 1977; Smith & Rozengurt, 1978*b*), inhibited 50% of the ouabain-sensitive influx, while ouabain-resistant influx was relatively resistant (Table 2).

Table 2. Selective inhibition of Rb influxes by various drugs[a]

Drug	Concentration (mM)	Rb influx activities (%)	
		Ouabain-sensitive	Ouabain-resistant
Ethacrinic acid	2	144	12.4
Amiloride	1	51.3	86.1
DNDS	0.1	173.4	97.4
Monensin	0.01	178.6	87.0

[a] Rb influxes were assayed on serum-stimulated cell cultures as described in Materials and Methods. Rb influxes were assayed in the presence of the indicated drug concentrations. Control activities (100%) were 18.1 and 11.8 pmol/min/μg protein for ouabain-sensitive and ouabain-resistant, respectively.

Table 3. Anion specificity of ouabain-resistant Rb influx[a]

Anion	Ouabain-resistant Rb influx (%)
Cl^-	100
NO_3^-	0
CO_3^-	0
CH_3COO^-	47

[a] NO_3^-, CO_3^- or CH_3COO^- (150 mM) were added as a major anion substituting Cl^-. The growing cultures were prewashed with isotonic solution (165 mM) of sodium or potassium salt of the indicated anion and then assayed as described in Materials and Methods. The ouabain-resistant Rb influx in the presence of Cl^- (100%) was 5.3 pmol/min/μg protein.

In contrast to amiloride, monensin is known as a Na ionophore, catalyzing exchange of H^+ and Na^+ and enhancing influx of Na into the cell (Pressman, 1976). The effect of monensin on ouabain-sensitive and resistant Rb influxes was tested and we found that it activated ouabain-sensitive Rb influx (Smith & Rozengurt, 1978*b*) but did not affect the ouabain-resistant one (Table 2).

Replacing Cl^- in the assay mixture by other anions such as NO_3^-, CO_3^- and CH_3COO^- resulted in high inhibition of ouabain-resistant Rb influx (Table 3). This might imply that the ouabain-resistant influx was a cotransport of K^+ (Rb^+) with Cl^-.

4,4′-dinitro-2,2′ stilbene disulfonic acid (DNDS) known as a specific inhibitor of the anion transport system of the red blood cell (Barzilay & Cabantchik, 1979) was found to be ineffective on the Rb influxes (Table 2). The fact that DNDS did not inhibit the ouabain-resistant influx indicated that it is a different carrier from the anion carrier (band III) of red blood cells (Barzilay & Cabantchik, 1979).

Discussion

Ouabain-resistant Rb influx has been studied in several cells such as erythrocytes, reticulocytes and Ehrlich ascites cells. This activity has been shown, by several groups, to catalyze Rb and K influx by a mechanism different to that of the Na/K-pump (Sachs et al. 1975; Tupper, 1975; Tupper et al. 1977; Bakker-Grunwald, 1978; Dunham, Steward & Ellory, 1980; Geck et al., 1980; Lauf & Theg, 1980; Panet and Atlan, 1980).

In this study we characterized the ouabain-resistant carrier-mediated Rb influx of NIH 3T3 fibroblasts, and compared the Rb influxes in quiescent and serum-stimulated cells. In the quiescent cultures only 10–20% of the total Rb influx was ouabain-resistant; however, in the serum-stimulated cells both influxes, ouabain-sensitive and ouabain-resistant, reached the same Rb influx rates per cell. Therefore, the extent of the stimulation by serum was higher for ouabain-resistant influx in comparison with that of the Na/K-pump. We were able to show that the activation of ouabain-resistant influx preceded that of the Na/K-pump. It appears, therefore, that the ouabain-resistant Rb influx is the earliest measurable event following serum addition to quiescent cells.

Two major modifications in the assay enabled us to measure accurately the two Rb influxes: (1) The high Rb concentration (5 mM) which saturated both influxes; and (2) the short assay time (2 min). The rapid activation of ouabain-resistant Rb influx by serum was followed by a fast decay and could therefore be noticed only by a short assay time. Similar results were also obtained when ^{86}Rb (5 mM) was added for 2 min directly to the cell culture medium which contains Mg^{++} and Ca^{++}. Removal of Ca^{++} and Mg^{++} from the assay mixture did not affect the ouabain-resistant influx, nor did it change its stimulation with the addition of serum (results not shown here). This is in accordance with results showing that Ca^{++} decreases passive diffusion of Rb and Na through cell membrane only, and has no effect on the Na/K-pump or furosemide-sensitive Rb fluxes (Wenner & Hackney, 1976). The possibility that different cell lines, growth conditions or slight differences in conditions to achieve quiescence was used by others (Rozengurt & Heppel, 1975; Smith, 1977; Tupper et al., 1977) as being a reason for not noticing the serum stimulation of ouabain-resistant Rb influx was not ruled out.

It has been recently proposed that growth-promoting factors activated the Na/K-pump by affect-

ing Na transport into the cells. Such increase enhanced the supply of internal Na to the Na/K-pump and as a result activated it (Smith & Rozengurt, 1978*b*). In order to investigate whether the ouabain-resistant Rb influx is in fact activated by the same mechanism, we tested two drugs; amiloride, which decreases cell membrane Na permeability (Bentley, 1968; Johnson et al., 1976; Sudou & Hoshi, 1977; Smith & Rozengurt, 1978*b*) and monensin, which stimulates it (Pressman, 1976). Both had no effect on the ouabain-resistant influx, appearing, thereby, that unlike the Na/K-pump the ouabain-resistant Rb influx is not mediated by the Na permeability of the cell membrane.

The ouabain-sensitive Rb influx has been studied by several groups in relation to the cell cycle (Mills & Tupper, 1976; Rozengurt & Mendoza, 1980). In this study we reported that ouabain-resistant influx was also modulated following the release of cells from the G_1/G_0 phase. A further study is needed to gain a better understanding of the role of these fluxes, in the control of cell proliferation.

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