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Na, K-ATPase pump in activated human lymphocytes: on the mechanisms of rapid and long-term increase in K influxes during the initiation of phytohemagglutinin-induced proliferation

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Abstract

Functional expression of Na, K-ATPase pump as determined by ouabain-sensitive Rb influxes has been investigated in human peripheral blood lymphocytes, activated by phytohemagglutinin (PHA) from resting state to proliferation. It is found that a rapid twofold elevation of ouabain-sensitive Rb influx in response to PHA is followed by a long-term increase in pump activity, which precedes the DNA synthesis and is temporally related to the growth phase of mitogenic response. Unlike the early pump activation, the late enhanced pump activity is not the result of elevated cell Na content, it is inhibited by cycloheximide and requires new protein synthesis. Actinomycin D and α-amanitin, in doses, which suppress the PHA-induced increase in the RNA synthesis, do not abolish the elevated Rb influx until 20–24 h of mitogenic activation and inhibit the late, growth-associated increase in Rb influx. It is concluded that (1) in mitogen-activated cells both short- and long-term control is involved in the enhanced pump activity, and (2) translational and transcriptional mechanisms may contribute to the long-term up-regulation of Na, K-ATPase pump during blast transformation of human lymphocytes.

Keywords: Na, K-ATPase pump; Proliferation; Cycloheximide; Actinomycin D; α-Amanitin; Human lymphocyte

1. Introduction

The Na, K-ATPase pump plays a 'housekeeping' role in maintaining low Na and high K concentrations in most of the animal cells and is central to the control of cell volume and membrane potential. In addition, the pump controls (although indirectly, by utilising Na gradient) transport of some nutrients, intracellular pH and free cytoplasmic Ca concentration. It is shown that the pump-mediated fluxes are rapidly increased in response to diverse growth-promoting stimuli [1–6]. The rapid pump activation in mitogen-stimulated cells has been considered mainly as secondary to an increase in the intracellular Na concentration due to the activation of Na–H exchange [3–8].

In addition to the early, transient activation of the Na, K-ATPase pump, the delayed, sustained increase in K influxes via pump has recently been found in

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phytohemagglutinin (PHA)-activated human lymphocytes during the later transit from resting state to proliferation [9]. The long-term increase in ouabain-sensitive K fluxes has also been reported for serum-stimulated fibroblasts and neuroblastoma cells in culture [10–12]. Until now, the functional meaning and mechanisms underlying the long-term activation of ion pump in mitogen-stimulated cells has not been defined. The available data led us to believe that during the cell cycle progression, the sustained activation of Na, K-ATPase pump is provided by mechanisms other than short-term regulation by internal Na concentration. Indeed, in our preliminary study we reported that in human lymphocytes, activated by PHA, an increase in the expression of α- and β-subunit mRNA of the Na, K-ATPase can take place as early as the 8th hour of mitogenic activation, thus indicating the possibility that the control of pump activity could occur at transcriptional or/and translational levels [13].

This study concerns the mechanisms responsible for the Na, K-ATPase pump activation during the development of mitogenic response. Primary T lymphocytes have proven to be a useful model for the study of proliferation-related events, mainly because of the availability of resting cells arrested in G_0, which can be induced to leave G_0, traverse G_1 and enter S phase by T-cell mitogens. First, we have examined the relations between K influxes and the transformation-associated events, such as the accelerated protein and RNA synthesis, the cell enlargement, the initiation of DNA synthesis in PHA-activated lymphocytes. Next, in order to define the nature of the elevated pump activity in activated lymphocytes, the inhibitors of translation and transcription were used. A preliminary account of the part of this work has recently been published [14]. Here, we present new evidence for the distinct regulation of Na, K-ATPase pump in early and long-term responses of cells to mitogens.

2. Materials and methods

2.1. Reagents and solutions

Culture media RPMI-1640 was obtained from flow. Phytohemagglutinin-M (PHA) was from Calbiochem. Ouabain, actinomycin D, α-amanitin and cycloheximide were obtained from Sigma. The stock solution of PHA was prepared in 0.15 M NaCl at a concentration 1.0 mg/ml. Ouabain and cycloheximide were dissolved in water at concentrations of 1.0 mM and 0.5 mg/ml, respectively. Stock solutions of actinomycin D and α-amanitin were prepared in ethanol. The ethanol concentration in the incubation medium did not exceed 0.1%. [^{3}H]thymidine, [^{3}H]uridine and [^{14}C]leucine were from Isotope, Russia. All other laboratory chemicals were Analytical Reagent grade from Reachim, Russia.

2.2. Cell preparation and culture conditions

Lymphocytes were isolated from fresh venous blood of healthy donors at Ficoll-verografin gradient centrifugation (1.077 g/ml, $400 \times g$), as described previously from our laboratory [9]. Briefly, isolated lymphocytes were washed thrice with Ca-free Hanks' balanced salt solution and resuspended in RPMI-1640 medium supplemented with glutamine (2 mM) and heat-inactivated AB IV Rh(+) serum (5%), without any antibiotics. After depletion of adherent cells on plastic dishes, lymphocytes were incubated overnight in glass bottles (10^8 cells/bottle in 50 ml medium) at 37°C with 5% CO_2. For activation experiments, at the next day after isolation the cell suspension was made up to a concentration of 1.5×10^6 cells/ml, distributed into small vials (2 ml suspension/vial) and PHA added to a final concentration of 20 μg/ml. The lymphocyte cultures thus prepared were incubated at 37°C with 5% CO_2 for the appropriate time intervals. Lymphocytes of one donor (up to 400×10^6 cells) were used in each experiment and experiments were repeated using lymphocytes isolated from blood of different donors to control the biological variation among donors.

2.3. K influx, intracellular K, Na and protein contents

Pump-mediated transport was assayed by measuring the ouabain-sensitive uptake of Rb, physiological analogue of K [15]. At definite time points throughout the experiment (0.5, 2, 5, 8, 16, 24, 48 and 72 h after PHA addition), RbCl at a final concentration of 2.5 mM was added to each incubation vial alone or

simultaneously with ouabain (final concentration 10^{-4} M). Incubations were continued for 30 min at 37°C in a humidified atmosphere containing CO_2. During this period of incubation, the rate of Rb uptake was linear and the net uptake of Rb was taken as a measure of the Rb flux into the cell. At the end of the incubation time, cell suspensions were carefully transferred into Eppendorf tubes and centrifuged once at $1000 \times g$ for 3 min. The cell pellets were rinsed five times with cold isotonic $MgCl_2$ without dispersion in order to avoid the disruption of cell aggregates, which are very compact at the initial stages of lymphocyte activation: the disturbance of lymphocyte aggregates was shown to induce a significant decrease in cellular K content [16]. Our preliminary experiments confirmed that the procedure using cell pellet washing without resuspension represents a reliable way to assess cellular cations (Rb, K, Na) without their loss from lectin-activated lymphocytes. The cell pellets were treated with 1 ml of 1% trichloroacetic acid (TCA) and TCA extracts were analysed for Rb, Na and K by emission flame photometry using a Perkin–Elmer AA 306 atomic absorption spectrophotometer. TCA precipitates were dissolved in 0.1 N NaOH and analysed for protein by Lowry procedure. The intracellular ion content was expressed as amount of ions per amount of protein in each sample analysed. Uptake of Rb related to Na–K pump was determined by substraction of the Rb amount taken up in the presence of 10^{-4} M ouabain from that in its absence. The Rb influxes thus estimated were expressed as micromoles per gram of protein for 30 min.

2.4. Protein, RNA and DNA syntheses

To assess the degree of PHA stimulation, cells were pulsed with [^{3}H]thymidine, [^{14}C]leucine or [^{3}H]uridine. At a definite time point during the activation experiment cell suspensions were incubated with [^{14}C]leucine (final radioactivity 2.7 μCi/ml) in combination with [^{3}H]thymidine (final radioactivity 10 μCi/ml) or with [^{3}H]uridine (final radioactivity 10 μCi/ml) for 30 min at 37°C with 5% CO_2. Incubations were terminated by centrifugation at $1000 \times g$ for 3 min. The same TCA precipitates, where intracellular cation and protein content were determined, were also analysed for radiotracers incorporation. The radioactivity of incubation medium and of TCA precipitates, dissolved in 0.1 N NaOH, was counted in a liquid scintillation counter incorporating a [^{3}H]/[^{14}C] dual channel analyser (Beckman LS 8100). The rate of radiotracer incorporation was calculated per gram cell protein. Three or five cultures were used at each time point of the activation experiment.

2.5. Cell size determination

The percentage of lymphocytes that undergo morphological blast transformation in response to PHA treatment was determined by analysing the volume distribution profile of lymphocyte populations with scanning microscope ‘Magiscan-2’ (Joyce-Loebl). At appropriate time of PHA stimulation, cell suspension was transferred into the tube, centrifuged at not higher than $1000 \times g$ for 3 min, rinsed with Ca-free Hanks’ balanced salt solution and disaggregated by pipette mixing. Thereafter, the cell suspension was placed on the plate and fixed using Giemsa technique with ethanol–formaline–acetic acid mixture. To calculate the volume of lymphocytes the cells on slides were approximated as ellipsoids. The volume distribution profile was obtained by measuring 200 cells in each preparation.

2.6. Statistics

All the data are presented as means ± S.E. for a certain number of experiments performed on lymphocytes from different donors. The number of experiments is indicated in each figure legend.

3. Results

3.1. Rb influx during the transition of human lymphocytes from resting state to the DNA synthesis

In resting lymphocytes the Rb influx at the external Rb concentration 2.5 mM accounts for 37.2 ± 2.0 μmol/g/30 min ($n = 25$). The major portion of this flux is inhibited by ouabain, thus representing the Na, K-ATPase pump-mediated flux (24.2 ± 3.5 μmol/g/30 min, $n = 24$). When freshly isolated

lymphocytes were incubated in RPMI medium supplemented with serum for two days, no significant changes in Rb influxes, both total and ouabain-sensitive, were detectable. However, on the third day, in cultures of lymphocytes without mitogens significant alterations in Rb transport do occur: ouabain-sensitive Rb influx decreases to 9.3 ± 2.9 μmol/g/30 min ($n = 4$) and residual, ouabain-resistent flux increases about twofold.

The application of PHA in the mitogenic concentration (20 μg/ml) to a suspension of resting lymphocytes results in an increase in Rb influx, mainly due to the increase in ouabain-sensitive component (Fig. 1). At least two stages in the PHA-induced elevation of the ouabain-sensitive Rb flux can be distinguished. The initial twofold increase during the first 2 h is followed by a plateau between 8 and 16 h, when the Rb influx is about twice as high as that in unstimulated cells. Later, a significant increase in the Rb influx is observed after 16 h. By 48 h the pump-mediated influx achieved a maximum value and then declines gradually toward the level in unstimulated cells. It should be noted that in this study, Rb fluxes represent the values calculated per gram of cell protein. When expressed per cell, the late increase in ouabain-sensitive Rb flux was much higher due to greater protein content in PHA-activated lymphocytes in comparison with the resting lymphocytes.

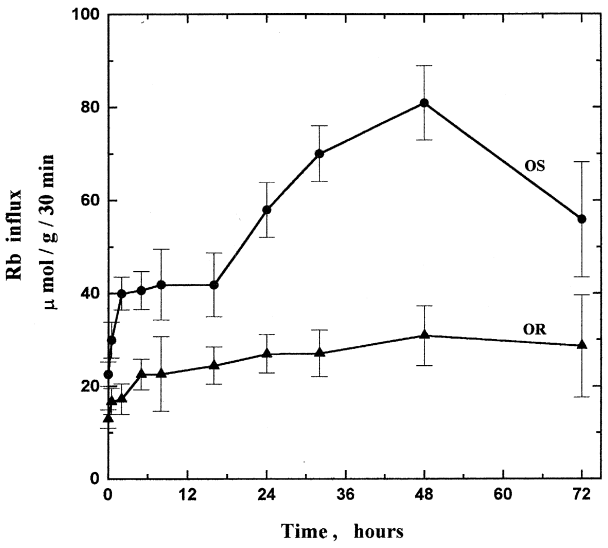

Fig. 1. Time course of ouabain-sensitive and ouabain-resistant Rb influxes in human lymphocytes activated by PHA. Isolated lymphocytes were incubated with PHA (20 μg/ml) for 0–72 h. At the definite time points RbCl (final concentration 2.5 mM) alone or with ouabain (final concentration 0.1 mM) was added to culture medium for 30 min and then cells were analysed for Rb uptake as described in Section 2. Ouabain-sensitive influx (OS, circles) was found as a difference between the total Rb uptake and the uptake in the presence of ouabain (OR, triangles up). All the values are means ± SD of 24 independent experiments, determined in triplicate for each time point in each experiment. Each activation experiment was performed on lymphocytes from one donor.

The ouabain-resistant Rb influx in PHA-activated lymphocytes increases from 12.9 ± 1.6 ($n = 22$) to 17.2 ± 1.1 μmol/g/30 min ($n = 19$) (with 2.5 mM RbCl in the external medium) by the end of the second hour of stimulation (Fig. 1). The flux continues to rise gradually during the later stages of mitogen action and by 48 h the ouabain-resistant Rb flux across the plasma membrane amounts to 28.1 ± 3.9 μmol/g/30 min ($n = 20$), thus comprising about 25% of the total Rb flux into lymphocytes.

In order to characterise the relationship between the ion flux changes and the initiation of cell proliferation we compared the pattern of changes in Rb influx with the basic proliferation-related events in activated lymphocytes. Data in Fig. 2(A) show that the rate of DNA synthesis, which peaked at approximately 48 h, is preceded by a major increase in RNA and protein synthesis. The mean cell volume of resting lymphocytes is increased about two times following PHA stimulation (Fig. 2(B)). The amount of cellular protein, a parameter that is closely correlated to cell size, is also increased during the second day of mitogenic stimulation (Fig. 2(B)). On comparing the time courses of ouabain-sensitive Rb influx with that of the macromolecular syntheses in PHA-stimulated lymphocytes we came to the conclusion that the late sustained activation of Na, K-ATPase pump precedes the DNA synthesis and is temporally related to the growth phase of mitogenic response.

3.2. Na, K-ATPase pump activation and intracellular Na content

Mitogens stimulate Na influx in most cells including human lymphocytes through an amiloride-sensitive Na/H exchange and it is generally believed that the rapid stimulation of the Na, K-ATPase pump in PHA-treated lymphocytes is due to an increase in

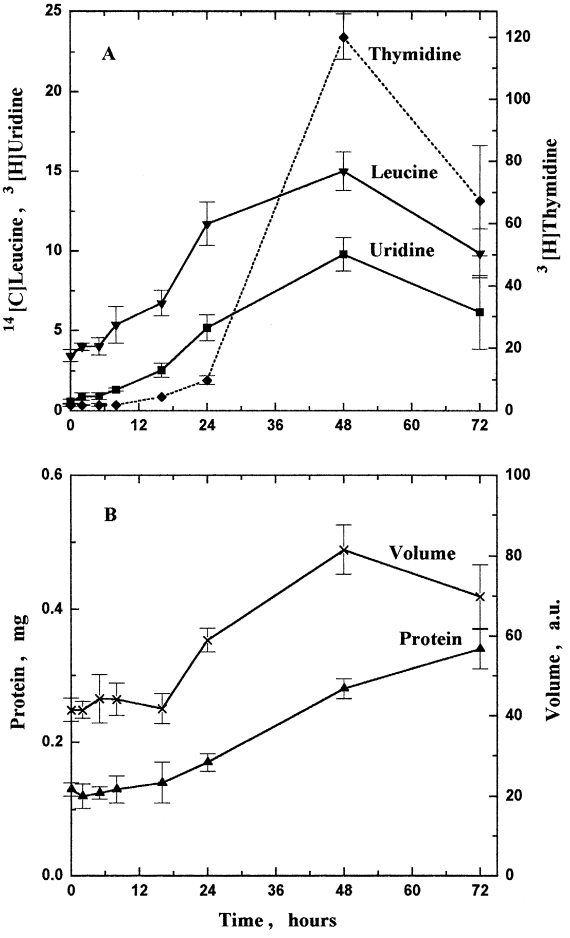

Fig. 2. Time course of protein, RNA and DNA syntheses (A), cell volume and total protein content (B) in human lymphocytes activated by PHA. The same lymphocyte cultures shown in Fig. 1 were used. Lymphocytes were incubated with PHA (20 μg/ml) for 0–72 h. (A) To examine the rates of macromolecular syntheses at the definite time points lymphocytes were pulsed for 30 min and analysed for 14[C]leucine (triangles down, left axis), 3[H]uridine (squares, left axis) and 3[H]thymidine (diamonds, right axis) incorporated into TCA-precipitable cell material. Incorporation rates are given as ($\times 10^3$) cpm/g/30 min. (B) Cell protein content (triangles up, left axis) was determined by Lowry method. Mean cell volume (crosses, right axis) was found from volume distribution profiles of cell populations, both resting and PHA-activated, as described in Section 2. All the values are means ± SD of 21–24 independent experiments, determined in triplicate for each time point in each experiment.

intracellular Na concentration (Na_i) [5,17–19]. To answer the question whether changes in Na_i could be responsible for the elevated fluxes via Na, K-ATPase pump in lymphocytes during $G_0/G_1/S$ transition, a comparison of ouabain-sensitive Rb influxes and Na_i should be made. As can be seen from Fig. 3, within the first hour of stimulation, Na_i increases from 141 ± 16.6 ($n = 24$) to 284 ± 33.0 μmol/g ($n = 21$), thereafter it decreases slightly and remains stable up to 48 h. The increase in Na_i is usually observed at 72 h, when the ouabain-sensitive Rb influx declines. These findings indicate that, at the early stage of PHA action, the elevated Na_i could indeed be the main cause of the rapid pump activation. On the contrary, the late sustained increase in pump-mediated K fluxes, which accompanies the development of growth phase in PHA-activated lymphocytes, can hardly be due to stimulation of Na, K-ATPase pump by the elevation in Na_i.

3.3. Protein synthesis and up-regulation of the Na, K-ATPase pump

To find out whether ongoing protein synthesis is required for the enhanced Na, K-ATPase pump in PHA-activated lymphocytes, cycloheximide (CHX),

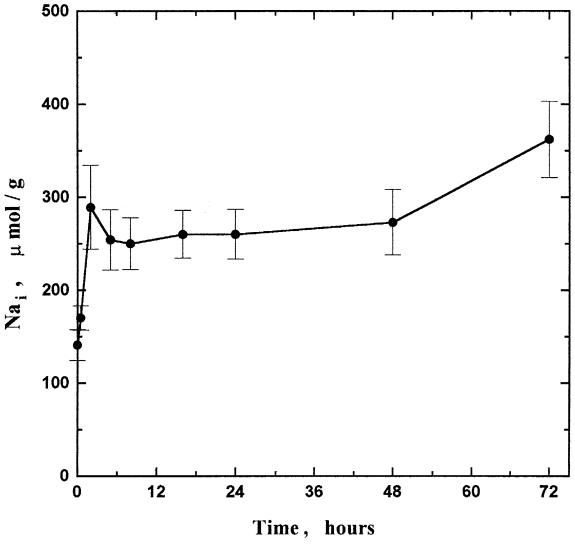

Fig. 3. Time course of sodium content in human lymphocytes activated by PHA. The same lymphocyte cultures shown in Fig. 1 were used. Isolated lymphocytes were incubated with PHA (20 μg/ml) for 0–72 h. At the definite time points of PHA stimulation lymphocytes were harvested for the determination of cell sodium content (Na_i) by flame emission photometry as described in Section 2. All the values are means ± SD of 24 independent experiments, determined in triplicate for each time point in each experiment.

Table 1
Effect of different concentrations of cycloheximide on [^{14}C]leucine incorporation, potassium and sodium content and ouabain-sensitive Rb influx in human lymphocytes, activated by PHA for 24 h

Cycloheximide (μg/ml)	[^{14}C]leucine incorporation ($\times 10^3$) (cpm/g/30 min)	Cation content (μmol/g)		Rb influx (μmol/g/30 min)	K_i/Na_i ratio
		K_i	Na_i		
0	14.36	678	158	49.0	4.3
0.5	3.46 (76.1)	657	101	53.8	6.5
5.0	2.76 (81.0)	655	122	55.7	5.4
10.0	1.20 (91.6)	500	200	50.1	2.5

Lymphocytes from blood of one donor were incubated with PHA (20 μg/ml) during 24 h and thereafter to some of the cultures cycloheximide at different concentrations was added. Following 2 h incubation with the drug, control (with PHA only) and cycloheximide-treated cultures were analysed for [^{14}C]leucine incorporation, potassium (K_i) and sodium (Na_i) content and ouabain-sensitive Rb influx as described in Section 2. Numbers in parenthesis indicate the per cent inhibition caused by cycloheximide. The data are the means (SD less 10%) of three independent cultures. The results of one representative experiment of three.

an inhibitor of translation, was used. Before applying CHX in long-term experiments, it was important to find those concentrations of the inhibitor, which suppressed protein synthesis and, at the same time, did not cause total cell damage in the course of experiment. Table 1 summarises the results of experiments with different CHX concentrations. The CHX concentration of 5 μg/ml was found to be more appropriate for our purposes: at 5 μg/ml CHX more than 80% of protein synthesis is inhibited within the first hour without a noticeable rapid changes in intracellular monovalent cations and Rb influx (Table 1). When present in culture of resting lymphocyte, 5 μg/ml CHX leads to an irreversible fall in Rb influxes and K_i/Na_i ratio as well as in the rate of RNA synthesis and protein content per culture after 16 h only, suggesting a total toxic effect of the drug (Table 2). In view of these results, in subsequent experiments CHX at a concentration of 5 μg/ml has been included into lymphocyte cultures for the time interval not longer than 12–16 h.

When added to the lymphocyte culture simultaneously with PHA, CHX (5 μg/ml) does not eliminate the initial increase of ouabain-sensitive Rb influx at

Table 2
Long-term effects of 5 μg/ml cycloheximide on protein and RNA synthesis, potassium and sodium content, Rb influx and total cell protein in resting human lymphocytes

Time (h)	Incorporation ($\times 10^3$) (cpm/g/30 min)		Cation content (μmol/g)		Rb influx (μmol/g/30 min)		Protein (mg)
	[^{14}C]Leucine	[^{3}H]Uridine	K_i	Na_i	OS	OR	
0	3.66	0.499	535	154	44.1	14.8	0.195
2	0.576	0.420	529	148	38.9	12.5	0.192
5	0.560	0.413	524	148	39.4	14.5	0.191
8	0.520	0.245	504	159	39.9	12.9	0.180
16	0.553	0.192	479	197	28.1	10.5	0.181
24	0.592	0.140	367	204	13.1	6.6	0.138

Lymphocytes from blood of one donor were incubated in RPMI medium supplemented with glutamine (2 mM) and heat-inactivated AB IV Rh(+) serum (5%) in the presence of 5 μg/ml cycloheximide. At definite time points the rate of [^{14}C]leucine and [^{3}H]uridine incorporations, intracellular potassium (K_i) and sodium (Na_i) content, Rb influxes, ouabain-sensitive (OS) and ouabain-resistant (OR) as well as total cell protein were determined as described in Section 2. Resting lymphocytes without cycloheximide were analysed simultaneously with 2 h drug-treated cultures. The data for each point are means (SD less 10%) of three independent cultures. The results of one representative experiment from three.

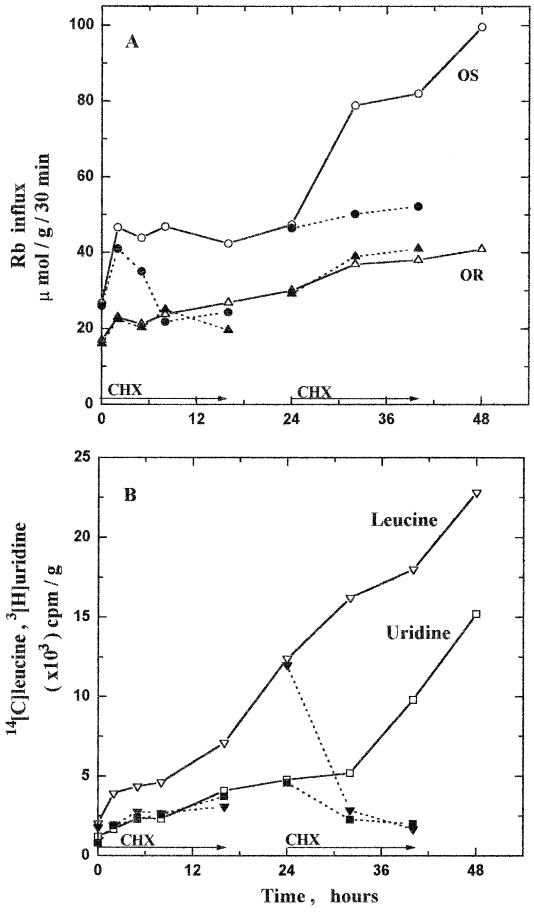

Fig. 4. Effect of cycloheximide on ouabain-sensitive and ouabain-resistant Rb influx (A) and protein and RNA synthesis (B) in PHA-activated human lymphocytes. Lymphocytes from blood of one donor were incubated with PHA (20 μg/ml) for 0–48 h and to some of the cultures cycloheximide (CHX, 5 μg/ml) was added at zero time or at 24 h of PHA action. At definite time points of PHA stimulation, in the same cultures ouabain-sensitive (OS, circles) and ouabain-resistant (OR, triangles up) Rb influxes as well as 14[C]leucine (triangles down) and 3[H]uridine (squares) incorporation were determined as described in Section 2. Open symbols represent data for cultures without CHX, filled symbols and dot lines – that in the presence of CHX. The arrow indicates the time period of incubation in the presence of CHX. Data are from one representative experiment of five. Each data point is the mean for triplicate cultures.

0.5–2 h (Fig. 4(A)). In the presence of CHX, the early elevated influx begins to decline after 2 h and returns to the resting value at about 8 h. As shown in Fig. 4(B), addition of CHX to lymphocytes at the 24th hour of PHA stimulation inhibits the PHA-induced rise in the leucine incorporation and prevents further increase in ouabain-sensitive Rb influx. CHX does not effect the time course of ouabain-resistant Rb influxes in activated cells. These findings indicate that the long-term enhancement of pump activity associated with the blastogenesis of lymphocytes requires new protein synthesis. It is important that, under these conditions, when protein and RNA synthesis are severely reduced, ouabain-sensitive Rb flux remains unaltered during 12–16 h of mitogenic activation (Fig. 4(A)).

3.4. Effect of inhibitors of transcription

We next examined the possibility whether transcriptional control might be involved in the regulation of the Na, K-ATPase pump in the course of blast transformation. Two inhibitors of transcription, actinomycin D and α-amanitin, were used. In view of multiple targets for these inhibitors in cell and possible general toxic effect, we first tested increasing concentrations of drugs on total RNA and protein synthesis and some parameters of ion transport, which

Table 3
Effect of different concentrations of actinomycin D on DNA synthesis, potassium and sodium content, volume and protein content in lymphocytes, activated by PHA

AD (μg/ml)	Time (h)	Thymidine (×10³) (cpm/g/30 min)	Cation content (μmol/g)		Volume (a.u.)	Protein (mg)
			K_i	Na_i		
0	0	0.7	648	141	0.36	0.089
	5	–	549	256	–	0.080
	24	4.5	708	235	0.60	0.109
	48	64.9	780	206	0.93	0.253
0.004	5	–	553	205	–	0.081
	24	1.1	709	183	0.48	0.094
	48	14.6	679	134	0.56	0.080
0.04	5	–	623	194	–	0.083
	24	0.8	413	164	0.45	0.071
	48	0.2	320	200	0.40	0.053

Lymphocytes from one donor were incubated with PHA (20 μg/ml) in the absence or presence of actinomycin D (AD) during 48 h at 37°C with 5% CO_2. Following 5, 24 or 48 h of incubation cells were analysed for [^{14}C]leucine, [^{3}H]uridine, [^{3}H]thymidine incorporation, potassium (K_i) and sodium (Na_i) content, volume and protein content as described in Section 2. Resting lymphocytes were taken for analysis simultaneously with 5 h-activated cultures. The results of one representative experiment of four. The data are means (SD less 10%) of three independent cultures.

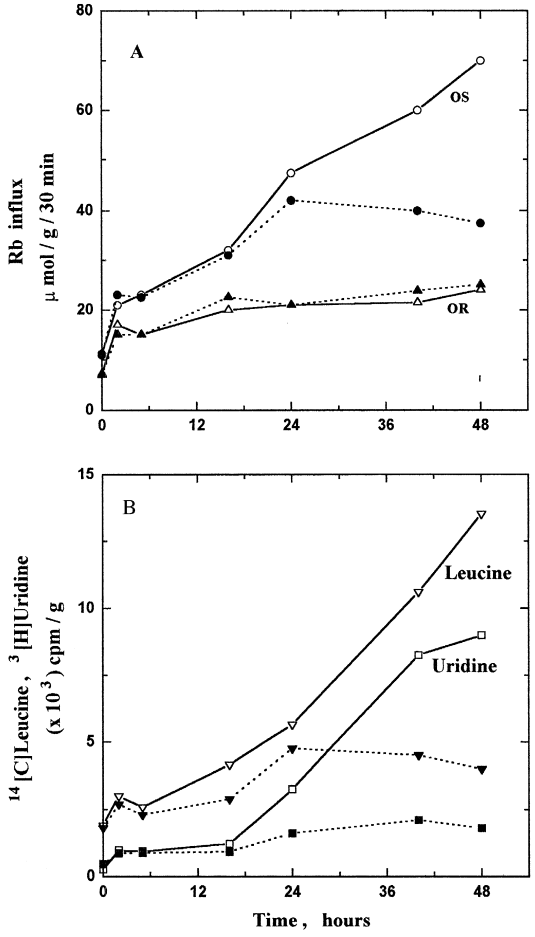

Fig. 5. Effect of actinomycin D on the time course of Rb influxes (A) and protein and RNA synthesis (B) in human lymphocytes activated by PHA. Lymphocytes isolated from blood of one donor were activated by PHA (20 μg/ml) in the absence or presence of actinomycin D (AD, 0.004 μg/ml) and at definite time points ouabain-sensitive (OS, circles) and ouabain-resistant (OR, triangles up) Rb influxes as well as 14[C]leucine (triangles down) and 3[H]uridine (squares) incorporation were determined as described in Section 2. Open symbols represent data for cultures without AD, filled symbols and dot lines – with AD. Data are from one representative experiment of nine. Each data point is the mean (SD less 10%) for triplicate cultures.

are sensitive indicators for cell survival. Our control experiments showed that actinomycin D at a concentration of 0.004 μg/ml is more appropriate for an examination of the long-term regulation of pump in lymphocytes, although higher concentrations (up to 0.04 μg/ml) can also be used in short-term experiments (Table 3). Actinomycin D at a concentration of 0.004 μg/ml, while partly inhibiting the initial rise in the RNA synthesis during the first day of mitogenic stimulation, suppressed the increase in [3H]uridine and [14C]leucine incorporation during the second day (Fig. 5(B)). This drug concentration blocked both the initiation of DNA synthesis and the enlargement of lymphocytes, however, cell K and Na

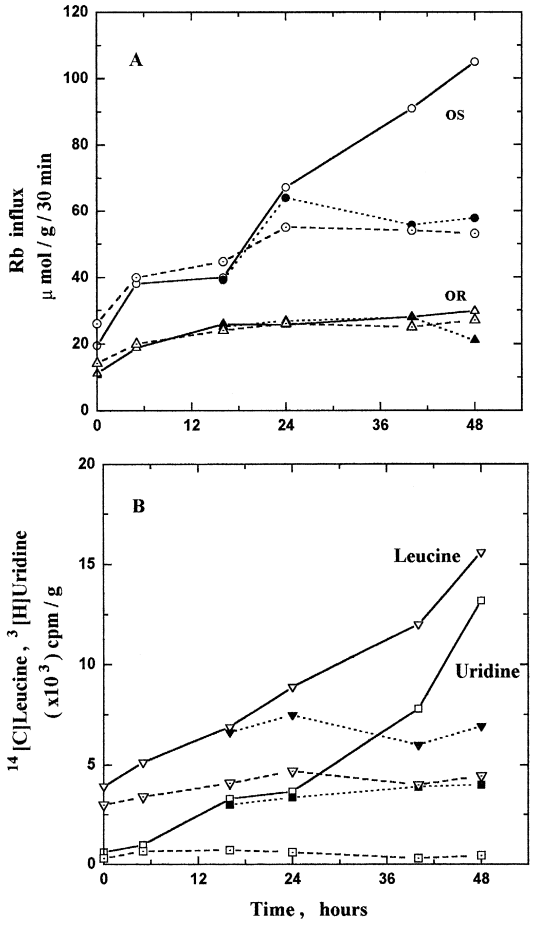

Fig. 6. PHA-induced changes of Rb influxes (A) and protein and RNA synthesis (B) in actinomycin D-pretreated lymphocytes and under the late actinomycin D treatment. The actinomycin D (AD) pretreatment: resting lymphocytes were incubated with AD (0.004 μg/ml) for 16 h and then stimulated by 20 μg/ml of PHA. The late AD treatment: lymphocytes were stimulated by PHA (20 μg/ml) and 16 h later some of the cultures received 0.004 μg/ml AD. At definite time points ouabain-sensitive (OS, circles) and ouabain-resistant (OR, triangles up) Rb influxes as well as 14[C]leucine (triangles down) and 3[H]uridine (squares) incorporation were determined as described in Section 2. Open symbols represent data for control cultures with PHA only, symbols with crosses and dashed lines – for AD-pretreated cultures, solid symbols and dot lines – for late AD-treated cultures. The experiment was performed on lymphocytes from one donor. Data are from one representative experiment of four. Each data point is the mean (SD less 10%) for triplicate cultures.

content were maintained near the level in unstimulated cells during the two days of experiment (Table 3).

When actinomycin D (0.04–0.004 μg/ml) was added to lymphocyte culture simultaneously with PHA, the elevation of ouabain-sensitive Rb influx within the first day was not affected (Fig. 5(A)). It was found that in the presence of actinomycin D the delayed increase in Rb flux was prevented after 24 h of PHA action. However, the degree of late flux inhibition was variable among different experiments. In this context, we revealed that while comparing the results utilising cells from different donors may be hazardous, results presented a consistent pattern when the time course of basic activation indices are examined in experiment on lymphocytes from one donor.

In our study, in lymphocytes from one donor the inhibition of the long-term enhancement of Na, K-ATPase pump in the presence of actinomycin D was correlated with the degree of inhibition of protein synthesis. When comparing the experiments on cells from different donors we came to the conclusion that during the first day of mitogenic activation the inhibition of the overall transcription by actinomycin D (as judged by [^{3}H]uridine incorporation) affects significantly neither the protein synthesis rate nor the stimulation of the Na, K-ATPase pump. After about 24 h, the long-term enhancement of cation pump is absent in case of suppression of protein synthesis in the presence of actinomycin D and is not enhanced above the level at 24 h (Fig. 5(B)).

Next, we studied effect of actinomycin D pretreatment on the PHA-induced activation of pump. In resting cells which were preincubated with 0.004 μg/ml actinomycin D for 16 h the rate of RNA synthesis was reduced by 60%, while the rate of [^{14}C]leucine incorporation, intracellular potassium and sodium content were not changed (data not shown). In actinomycin-treated lymphocytes, PHA did not enhance both total RNA and protein synthesis to any significant degree (Fig. 6(B)). It was revealed that pretreated cells were capable of increasing ouabain-sensitive Rb influx during the first 20 h after PHA addition, however, the flux elevation between 24 and 48 h was eliminated (Fig. 6(A)). Thus, pretreatment experiments have shown that during the first day of mitogenic activation of lymphocytes the pump-mediated cation transport can be stimulated at the suppressed RNA synthesis. This is not the case on the second day of activation.

To further define the period when transcriptional activity was required for the increased pump induction, actinomycin D was added at intervals from 16 to 48 h after initial exposure to PHA. In this type of experiments the acceleration of [^{3}H]uridine and [^{14}C]leucine incorporation between 24 and 48 h was

Table 4
Effect of α-amanitin on pump-mediated Rb influx, potassium and sodium content, protein, RNA and DNA syntheses, cell volume and protein content in human lymphocytes, activated by PHA

Incubation	Time (h)	Rb influx (μmol/g/30 min)	Cation content (μmol/g)		Incorporation ($\times 10^3$) (cpm/g/30 min)			Volume (a.u.)	Protein (mg)
			K_i	Na_i	Uridine	Leucine	Thymidine		
PHA									
	0	24.6	586	118	0.4	2.9	0.1	0.43	0.12
	24	53.0	617	290	4.3	12.1	3.8	0.60	0.13
	48	89.0	820	287	11.4	15.4	87.1	1.23	0.21
PHA + α-amanitin									
	24	53.1	556	294	4.0	12.0	3.1	0.54	0.11
	48	54.3	564	280	6.1	9.0	16.7	0.69	0.16
PHA 20 h, then PHA + α-amanitin									
	24	54.0	622	283	5.0	12.7	4.3	0.66	0.14
	48	59.7	634	287	8.7	9.8	9.8	0.87	0.17

α-Amanitin (4 μg/ml) was added to lymphocyte cultures simultaneously with PHA (20 μg/ml) or 20 h after PHA. Following 24 and 48 h after PHA induction cells were analysed for [^{14}C]leucine, [^{3}H]uridine and [^{3}H]thymidine incorporation, potassium (K_i), sodium (Na_i), protein content and volume as described in Section 2. The data are means (SD less 10%) of 3–5 cultures of lymphocytes from one donor. The results of one representative experiment from four.

absent and the elevation of the ouabain-sensitive Rb influx was abolished immediately after 8–10 h of drug application (Fig. 6). These data confirm the above observation that the inhibitory effect is dependent on the time of drug application: actinomycin D is effective in inhibiting the elevated pump during the second part of the $G_0/G_1/S$ transition rather than at the early stages of lymphocyte activation.

Another inhibitor of transcription, α-amanitin, produced effects which were virtually similar to those obtained with actinomycin D. Addition of α-amanitin at a concentration of 4 μg/ml simultaneously with PHA prevented elevation of the Rb influx during the second day of stimulation, but did not affect the flux increase up to 24 h of mitogen action (Table 4). The delayed inhibitory effect of α-amanitin was also evident in pretreatment experiments, when the drug was added to the cell culture 20 h prior to the PHA addition (data not shown). When applied after 20 h of PHA stimulation, α-amanitin abolished the late increase in pump-mediated Rb influx (Table 4). In all experiments performed the inhibitor suppresses the PHA-induced DNA synthesis and blocks the enlargement of cells. It is worth noting that α-amanitin, a specific inhibitor of RNA polymerase II, blocks a smaller percentage of the total RNA and protein synthesis than the less selective drug, actinomycin D.

4. Discussion

Mitogen-induced changes in pump-mediated K fluxes has been shown in lymphocytes from various sources [15,17–22]. However, most of the investigators have been confined to early stages of cell activation. In this study, we have shown that the mitogenic activation of human peripheral blood lymphocytes by PHA is accompanied by a long-term enhancement of the Na, K-ATPase pump. The elevated K influxes via pump are typical for the later stages of blastogenic transformation being temporally associated with the growth phase of $G_0/G_1/S$ transit.

Several mechanisms have been proposed to explain an increase in the Na, K-ATPase activity in mitogen-stimulated lymphocytes. First, the rise in the internal Na concentration has been suggested to be a cause for the increase in pump-mediated fluxes during the first minute of mitogen action [17]. Second, recruitment of plasma membrane pumps from an internal silent pool of inactive pumps has also been suggested for the upregulation of ion pumps between 2 and 5 h of the pig lymphocyte activation [22].

Our studies of ouabain-sensitive K fluxes indicate that in PHA-activated lymphocytes several phases in the pump response to mitogen should be distinguished based on the dependence on intracellular Na concentration and on effects of agents inhibiting protein synthesis and transcription. In the early mitogenic reaction of cell, a rapid increase in ion pumping appears to be due to the elevation of intracellular Na concentration. This increase can be brought about by changes in the turnover rate of existing Na/K pumps and by an increase in the number of active pumps in plasma membrane [22,23]. These mechanisms are independent of new protein synthesis. As shown in this study, the long-term activation of Na, K-ATPase pump that is apparent after many hours of mitogen application is not the result of an increase in internal Na content. It is abolished in the presence of cycloheximide immediately after 5–8 h of mitogen action, thus indicating that the sustained increase in pumping is dependent on a new protein synthesis. It is obvious that the data obtained do not allow to specify which of the newly synthesised proteins is critical for the sustained pump enhancement in activated cells: whether it is Na, K-ATPase α and/or β subunit proteins or some kind of a regulatory proteins. If we assume that it is the Na, K-ATPase itself, than the lifetime of Na, K-ATPase pump could be estimated as 16 h or more for both resting and activated lymphocytes. This value is compatible with the earlier estimations of half-life for the enzyme, although for other cells made [23,24].

From our studies of ion flux activation in the presence of inhibitors of transcriptions it follows that the protein synthesis-dependent increase in the Na, K-ATPase activity can be divided into two phases: actinomycin D (α-amanitin)-insensitive phase (between 5 and 20 h) is followed by actinomycin D (α-amanitin)-sensitive phase after about 20 h of PHA stimulation. The latter phase coincides temporally with the enlargement of activated lymphocytes.

The observed time dependence of the inhibitory effect of actinomycin D and α-amanitin on PHA-induced pump stimulation seems to be consistent with the peculiarities of activation process in quiescent

cells: in lymphocytes like in other dormant cells at the initial stage of mitogenic activation protein synthesis is mostly controlled at the translational level [25–27]. It might be proposed that during the first day of mitogenic activation the newly synthesised pump proteins which are likely to be responsible for an increase in ion fluxes are translated from the pre-existing Na, K-ATPase mRNAs, whereas at later cell cycle progression the generation of new pumps is also regulated at the transcriptional level.

All these assumptions are based on the studies at cellular level. Indeed, there are potential problems in using inhibitors of transcription at the cellular level to establish that a certain cellular response requires the new mRNA synthesis. The response might be blocked by non-specific action of the drugs. In the present study, we have addressed the problem by using drug concentrations as low as possible and by application of two transcriptional inhibitors with different modes of action. The fact that both inhibitors have suppressed the long-term increase in ion pumping is in favour of the suggestion that the suppression is due to the inhibition of mRNA synthesis. In the experiments presented, a non-specific effect of drugs seems to be minimal, since none of the drugs altered both the intracellular cation content and the rate of the [^{14}C]leucine incorporation over 24 h of treatment of resting lymphocytes. Moreover, in drug-pretreated cells the elevation of ouabain-sensitive K fluxes during the first day of PHA stimulation was found to persist.

Thus, the present study raises the possibility that in activated lymphocytes, the growth-associated activation of Na, K-ATPase pump may be controlled via genomic pathway. Few data are available on the changes in transcript level of Na, K-ATPase pump in proliferating cells as compared to quiescent cells. Proliferation-related changes in α_1 and β_1 subunit mRNAs have been reported for the regenerating rat liver and for a serum-depleted-restored liver cell line [28–31]. In our preliminary study, we found that the induction of mitogenic response in human lymphocytes is accompanied by an elevated level of Na, K-ATPase mRNAs as early as at 8 h of PHA stimulation, i.e. well ahead of the growth-related enhancement of pump activity [13]. We suggest as a working hypothesis that these early changes in the level of pump subunits mRNAs could be realised in the specific protein synthesis and in increased pump pool during the later stages of cell cycle transit. More studies are needed to relate the data emerging from both physiological and molecular approaches.

In conclusion, at present, it is not clear what the relationship is between ion pump activation and mitogenesis. Previous studies of monovalent cation fluxes in mitogen-stimulated cells gave evidence for the notion that an early activation of ion pump was not obligatory for the initiation of cell proliferation. In this study, we demonstrate that in PHA-activated human lymphocytes the elevated pumping and increased K influxes precede the proliferation and is parallel to the growth of small lymphocytes to blast cells. It has been postulated, that the cellular events, which are controlled by genes and occur primarily during G_1 phase, regulate cell cycle progression in mitogen-induced cells [32,33]. The ion pump is known to be implicated in the regulation of ion and osmotic balance in animal cells and provides the driving force for Na-coupled transport processes. It is conceivable that the enhanced Na, K-ATPase pump could somehow be involved in the genesis of growth response to mitogens, thus participating in cell cycle regulation at the physiological level.

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References

[1] J.G. Kaplan, Annu. Rev. Physiol. 40 (1978) 19–41.
[2] H.L. Leffert, K.S. Koch, in: A.L. Boynton, W.L. McKeehan, J.F. Whitfield (Eds.), Ions, Cell Proliferation and Cancer, Academic Press, New York, 1982, pp. 103–130.
[3] E. Rozengurt, Science 234 (1986) 161–166.
[4] W.H. Moolenaar, Annu. Rev. Physiol. 48 (1986) 363–376.
[5] S. Grinstein, S.J. Dixon, Physiol. Rev. 69 (1989) 417–481.
[6] A.A. Vereninov, I.I. Marakhova, Ion Transport in Cultured Cells, Nauka, Leningrad, 1986, p. 292.
[7] J.B. Smith, E. Rozengurt, Proc. Natl. Acad. Sci. U.S.A. 75 (1978) 5560–5564.
[8] E. Rozengurt, Adv. Enzymol. Regul. 19 (1981) 61–85.
[9] A.A. Vereninov, E.V. Gusev, O.M. Kazakova, E.M. Klimenko, I.I. Marakhova, V.V. Osipov, F.V. Toropova, Cytology 33 (1991) 78–93.

[10] J.T. Tupper, F. Zorgniotti, B. Mills, J. Cell. Physiol. 91 (1977) 429–440.
[11] C.L. Mummery, J. Boonstra, P.T. van der Saag, S.W. de Laat, J. Cell. Physiol. 107 (1981) 1–9.
[12] I.I. Marakhova, T.A. Vinogradova, E.V. Yefimova, Gen. Physiol. Biophys. 8 (1989) 273–282.
[13] A.A. Vereninov, I.I. Marakhova, V.V. Osipov, F.V. Toropova, FEBS Lett. 316 (1993) 37–40.
[14] I.I. Marakhova, A.A. Vereninov, T.A. Vinogradova, F.V. Toropova, FEBS Lett. 368 (1995) 110–112.
[15] G.B. Segel, B.R. Gordon, M.A. Lichtman, M.M. Hollander, M.R. Klemperer, J. Cell. Physiol. 87 (1976) 337–344.
[16] G.B. Segel, M.A. Lichtman, M.M. Hollander, B.R. Gordon, M.R. Klemperer, J. Cell. Physiol. 88 (1976) 43–48.
[17] K.V.S. Prasad, A. Severini, J.G. Kaplan, Arch. Biochem. Biophys. 252 (1987) 515–525.
[18] J.G. Kaplan, T. Owens, Annu. N.Y. Acad. Sci. 339 (1980) 191–200.
[19] G.B. Segel, M.A. Lichtman, J. Clin. Invest. 58 (1976) 1358–1369.
[20] R. Averdunk, P.K. Lauf, Exp. Cell Res. 93 (1975) 331–342.
[21] I.M. Redondo, A. Lopez-Rivas, M. Fresno, FEBS Lett. 206 (1986) 199–202.
[22] A. Severini, K.V.S. Prasad, A.F. Almeida, J.G. Kaplan, Biochem. Cell. Biol. 65 (1987) 95–104.
[23] T.A. Pressley, J. Membrane Biol. 105 (1988) 187–195.
[24] T.A. Pressley, R.S. Haber, J.N. Loeb, I.S. Edelman, F. Ismail-Beigi, J. Gen. Physiol. 87 (1986) 591–606.
[25] R.E.H. Wettenhall, D.R. London, Biochem. Biophys. Acta. 349 (1974) 214–225.
[26] H.L. Cooper, R. Braverman, J. Cell. Physiol. 93 (1977) 213–226.
[27] D.A. Hume, M.J. Weidemann, in: Research Monographs in Immunology, vol. 2, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, Oxford, 1980, pp. 1–232.
[28] A. Bhutada, C. Perez, D.Y. Chon, F. Ismail-Beigi, Am. J. Physiol. 252 (1990) C1044–C1050.
[29] A. Bhutada, W.W. Wassynger, F. Ismail-Beigi, J. Biol. Chem. 266 (1991) 10859–10866.
[30] X.P. Lu, H.L. Leffert, J. Biol. Chem. 266 (1991) 9276–9284.
[31] F.R. Simon, J. Fortune, A. Alexander, M. Iwahashi, R. Dahl, E. Sutherland, J. Biol. Chem. 271 (1996) 24967–24975.
[32] R. Baserga, Exp. Cell Res. 151 (1984) 1–5.
[33] A.B. Pardee, Science 246 (1989) 603–608.