Functional expression of the Na/K pump is controlled via a cyclosporin A-sensitive signalling pathway in activated human lymphocytes

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Abstract An immunosuppressant cyclosporin A (CsA) inhibits T-cell proliferation by blocking the nuclear factor of activated T-cells (NFAT) required for expression of the interleukin-2 (IL-2) gene. This work has demonstrated for the first time that in human blood lymphocytes (HBLs) activated by phytohemagglutinin (PHA), CsA at anti-proliferative doses inhibits the late sustained increase in ouabain-sensitive Rb(K) influxes, which accompanies the growth phase of G_0/G_1/S transition. CsA affects neither the initial, transient activation of the pump in response to PHA nor the ouabain-resistant ion fluxes during cell cycle progression. When the HBLs were rendered competent to proliferate by phorbol 12,13-dibutyrate ester and ionomycin in the presence of CsA, the exogenous IL-2 did not bypass the initial inhibitory effect of CsA on the long-term pump enhancement. When applied after the competence induction, CsA produced no effect on the sustained increase in ouabain-sensitive Rb influxes during the IL-2-induced progression phase. These results indicate that in activated HBLs, (1) IL-2 is involved in functional expression of the Na/K pump during cell transition from quiescence to proliferation, (2) the cell cycle-associated upregulation of the pump is related to a CsA-sensitive signalling pathway.

Key words: Na/K pump; K influx; Cyclosporin A; Interleukin-2; Human lymphocyte; Proliferation

1. Introduction

The key plasma membrane transporter in animal cells is the Na,K-ATPase pump which is responsible for the maintenance of electrochemical Na and K gradients across the plasma membrane. In the cell, the Na/K pump is under control of hormones and various stressor and growth-promoting factors that impart both a short-term and long-term regulation of the pump activity [1–5]. Most data on the growth factor-related pump regulation deal with rapid transient modulations of K influxes in response to mitogens. This response is mainly provided by changes in the turnover rate of pre-existing pumps and/or redistributions of silent pumps between the subcellular compartments and the cell surface [4–6]. The precise mechanisms which control the sustained enhancement of the pump during the later stages of cell transition from the resting state to proliferation have not been defined. We have recently demonstrated that in phytohemagglutinin (PHA)-induced human blood lymphocytes (HBLs), a long-term increase in pump activity accompanying the blast transformation could not be regulated by short-term mechanisms including an increase in the intracellular Na^+ concentration [7]. Moreover, the growth-associated activation of the pump was found to be dependent on a new protein synthesis and could be controlled at the transcriptional level [7,8]. All the available data permit us to suggest that in mitogen-induced cells, a long-term control of the Na/K pump should be mediated by specific genes whose activation is somehow linked to the whole genomic program of initiating cell proliferation. The goal of this study was to find out whether the functional expression of the Na/K pump during the cell cycle transition is linked to initial steps of lymphocyte activation.

Cyclosporin A (CsA), a potent immunosuppressant, inhibits T-cell proliferation by suppressing transcription of a T-cell growth factor, interleukin-2 (IL-2) [9,10]. The inhibitory effect of CsA is due to inactivation of calcineurin, Ca^{2+}-dependent phosphatase, that activates the nuclear factor of activated T-cells (NF-AT) required for expression of the IL-2 gene [10–13]. Studies with native cells have shown that CsA is effective in the inhibition of proliferative responses when applied at the initial stage of mitogenic stimulation and does not block the induction of cell proliferation during the progression phase to DNA synthesis [14,15]. We used such a peculiarity of CsA of inhibiting selectively the IL-2 production to elucidate relations between the sustained activation of the Na/K pump and the CsA-sensitive signalling pathway for IL-2 expression. This paper presents for the first time evidence that CsA in doses which inhibit the induction of HBL proliferation eliminates the long-term enhancement of the Na/K pump activity during cell transition from the resting state to DNA synthesis.

2. Materials and methods

HBLs were purified from fresh venous blood by Ficoll-verografin gradient centrifugation (1.077 g/ml, $400\times g$) [7]. After depletion of adherent cells on plastic bottles, isolated HBLs were incubated overnight in RPMI 1640 medium (Flow) with heat-inactivated AB IV Rh(+) serum (5%). At the next day, the cell suspension (1.5×10^6 cells/ml) was placed into vials (2 ml suspension/vial) and PHA (Calbiochem), alone or with CsA (a gift from Sandoz, Switzerland), was added to a final concentration of 20 μg/ml. Then, the cultures of HBLs, both resting and PHA-activated, were incubated at 37°C in a humidified atmosphere with CO_2 and at the appropriate time points throughout the experiment, the cultures were taken for cation determinations. Ouabain-sensitive Rb uptake was measured to evaluate activity of the Na/K pump. RbCl (2.5 mM), alone or with ouabain (0.1 mM, Sigma), as well as [^{3}H]thymidine, [^{14}C]leucine or [^{3}H]uridine (Isotope, Russia), were added to HBL cultures and after 30 min incubation at 37°C with CO_2, the cell suspensions were transferred to Eppendorf tubes, centrifuged once at $1000\times g$ for 3 min and the pellets were rinsed five times with cold isotonic $MgCl_2$ without dispersion to avoid disruption of cell aggregates which are very compact at the initial stages of PHA activation. The cell pellets were treated with 1 ml of 1% trichloroacetic acid (TCA) and TCA extracts were analyzed for Rb, Na and K by emission flame photometry using a

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Perkin-Elmer AA 306 atomic absorption spectrophotometer. TCA precipitates were dissolved in 0.1 N NaOH and analyzed for protein by the Lowry procedure. Radioactivity of TCA precipitates dissolved in 0.1 N NaOH was counted in a liquid scintillation counter incorporating a [^{3}H]/[^{14}C] dual channel analyzer (Beckman LS 8100). The intracellular ion content as well as the rates of radiotracer incorporations were calculated per g cell protein. Three or five cultures were used for each time point of the activation experiment. Lymphocytes from one donor (up to 400×10^6 cells) were used in each experiment. The percentage of lymphocytes that undergo morphological blast transformation in response to PHA treatment was determined by analyzing the volume distribution profile of lymphocyte populations with a scanning microscope 'Magiscan-2' (Joyce-Loebl).

To induce 'competence', HBLs were stimulated either by a combination of 10 nM phorbol 12,13-dibutyrate ester (PDB, Sigma) and 500 nM ionomycin (Sigma) or by 2 μg/ml PHA for 30–60 min. The cells were then washed twice in RPMI medium and placed into vials (3×10^6 cells/vial) in the complete medium. Thereafter, the activated HBLs received 200 U/ml recombinant IL-2 (Amersham) or a mitogenic concentration of PHA (20 μg/ml) to induce progression of competent lymphocytes to DNA synthesis.

All the data are presented as means ± S.E.M.

3. Results

3.1. Effect of CsA on pump-mediated Rb fluxes in PHA-activated lymphocytes

In PHA-activated HBLs, a rapid 2-fold increase in the ouabain-sensitive Rb influx after PHA addition is followed by a sustained elevation of the Rb influx between 16 and 48 h, which is parallel to cell enlargement and is closely associated with blast transformation [7]. As shown in Fig. 1, when added simultaneously with PHA, CsA (1 μg/ml) did not affect the initial rise in the ouabain-sensitive Rb influx in response to PHA. However, CsA was revealed to totally inhibit the Rb influx elevation via the Na/K pump during later mitogenesis. In activated HBLs, at 48 h, the ouabain-sensitive Rb influx increased about three times, whereas in the presence of CsA, the flux remained unchanged within 16 and 48 h. In contrast, the time course of ouabain-resistant Rb influxes was not affected by CsA both at the initial step of activation and during the progression to DNA synthesis (Fig. 1).

The inhibitory effect of CsA on pump-mediated transport is usually observed at the anti-proliferative doses of the drug. In experiments presented in Fig. 1, CsA at a concentration of 1 μg/ml inhibited the PHA-induced proliferation in HBLs, as judged by both a decrease in label incorporations into protein, RNA and DNA and an inhibition of HBL enlargement during G_0/G_1/S transition (Fig. 2). At the maximum proliferative response (48 h) in the presence of CsA, the [^{14}C]leucine, [^{3}H]uridine and [^{3}H]thymidine incorporations were decreased by 49.8 ± 4.3, 67.6 ± 10.1 and $60.4\pm5.3\%$, respectively. As seen from Fig. 2A, CsA blocked partially the HBL proliferation in response to PHA. The incomplete inhibition of the PHA-induced mitogenic response of HBLs appears to be due to the additional signalling pathways of IL-2 gene expression which is resistant to immunosuppressive drugs including CsA [16,17]. Fig. 2B also shows that in the constant presence of CsA, there also was an inhibition of the increase in intracellular potassium content (K_{in}), typical for the successful transition of resting HBLs to proliferation [18]. It should be emphasized that the long-term (for 48 h) incubation of the non-stimulated HBLs in the complete RPMI medium in the presence of CsA (1 μg/ml) affects neither the basal rates of protein and RNA synthesis nor the intracellular K and Na content (data not shown).

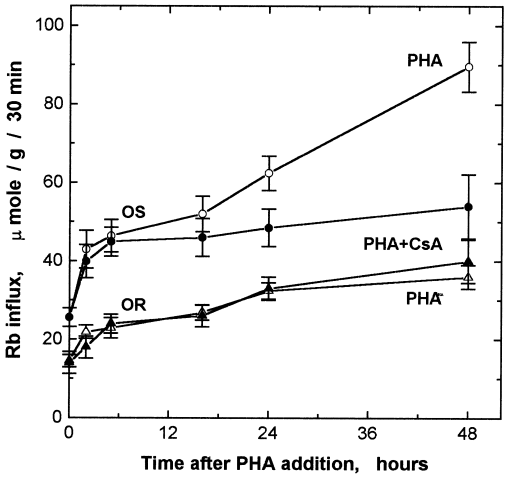

Fig. 1. Effect of CsA on the time course of ouabain-sensitive (OS) and ouabain-resistant (OR) Rb influxes in HBLs, activated by PHA. Open symbols, without CsA; closed symbols, with CsA (1 μg/ml). The data are means of 12 experiments.

3.2. CsA does not affect the cell cycle-associated pump activation in competent HBLs

Activation of the T-cell receptor (TCR) by antigens or mitogenic lectins initiates a variety of signalling pathways to activate early transcriptional events and eventually to induce the IL-2 signalling pathway responsible for the cell cycle progression and the entry into the S phase [19]. This complicated, multi-step cellular response can be experimentally divided into two separate phases. Initially, the TCR activation causes a transition from the resting G_0 state to the G_1 phase, so that the cell becomes competent to express the high-affinity IL-2 receptor (IL-2R) and to produce IL-2 [13,14]. Later on, cell cycle progression occurs after the required level of IL-2R has been occupied by IL-2.

It has been shown that after a brief exposure to PHA at a submitogenic concentration, HBLs do not proliferate. However, this treatment induces the IL-2 mRNA expression (cells acquire 'competence') and to enter the S phase and complete the cycle, PHA at a mitogenic concentration should be given [14]. Under this activation procedure, CsA inhibits the expression of IL-2 when given before or simultaneously with submitogenic PHA, whereas CsA does not prevent the IL-2 mRNA expression as well as IL-2 production during the later cell cycle progression of competent HBLs to DNA synthesis [14,15].

As shown in Fig. 3, if the competence was first induced by submitogenic PHA (2 μg/ml), then during the entire second incubation with a mitogenic concentration of PHA (20 μg/ml), CsA affected neither the late increase in the ouabain-sensitive Rb influx nor the increase in intracellular K content during the pre-replicative phase as well as the growth response and DNA synthesis in HBLs (Fig. 3 and Table 1). When CsA was present during the initial phase of competence induction with small doses of PHA, both the late increase in the ouabain-sensitive Rb influx and the elevated DNA synthesis at 48 h were suppressed (Table 1). These findings allow us to suggest that the induction of a long-term activation of the Na/K pump during the progression phase of G_0/G_1/S transition is

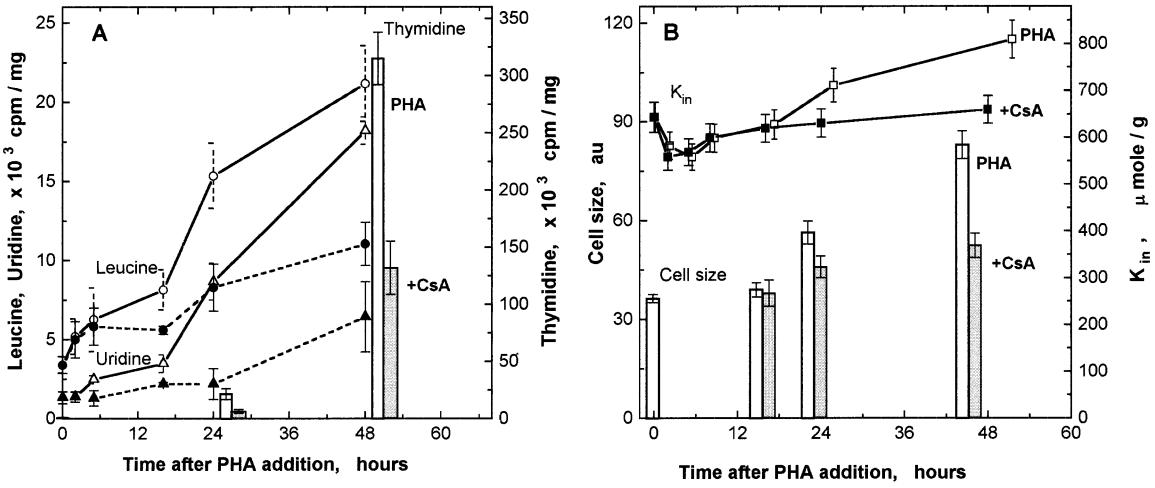

Fig. 2. Effect of CsA on the PHA-induced DNA, RNA, protein syntheses (A), cell growth and the time course of the intracellular K content (B) in HBLs. Open symbols and solid lines, without CsA; closed symbols and dashed lines, with CsA (1 μg/ml). CsA was given simultaneously with PHA (20 μg/ml). Macromolecular syntheses were determined as [^{3}H]thymidine (DNA), [^{3}H]uridine (RNA) and [^{14}C]leucine (total cell protein) incorporations.

somehow related to the initial, CsA-sensitive steps of mitogen induction in HBLs.

To elucidate the mechanism of the CsA-related inhibition of the ion pump in HBLs further, we applied exogenous IL-2. A brief 30 min incubation with the phorbol ester PDB and calcium ionophore, ionomycin, is sufficient to induce the state of competence in T-cells, including expression of functional IL-2Rs [13–15]. The addition of CsA during the initial 30 min incubations with PDB and ionomycin completely blocked both the expression of IL-2R mRNA and the mitogenic response to exogenous IL-2 [14]. On the contrary, if the competence was induced by PDB and ionomycin, addition of IL-2 together with CsA in the progression phase initiated the proliferative response.

As shown in Fig. 3, in the competent cells, after a treatment with PDB and ionomycin and with IL-2 added after washing the pretreated cells with RPMI medium, at 48 h, the ouabain-sensitive Rb influx reached the level which was comparable with the Rb influx in the PHA-stimulated HBLs. When CsA was present during the first 30 min induction of the competence by PDB and ionomycin, the cells failed to increase the pump fluxes during the second incubation with IL-2. When CsA was present during the second incubation only, IL-2 was found to induce sustained elevation of the ouabain-sensitive

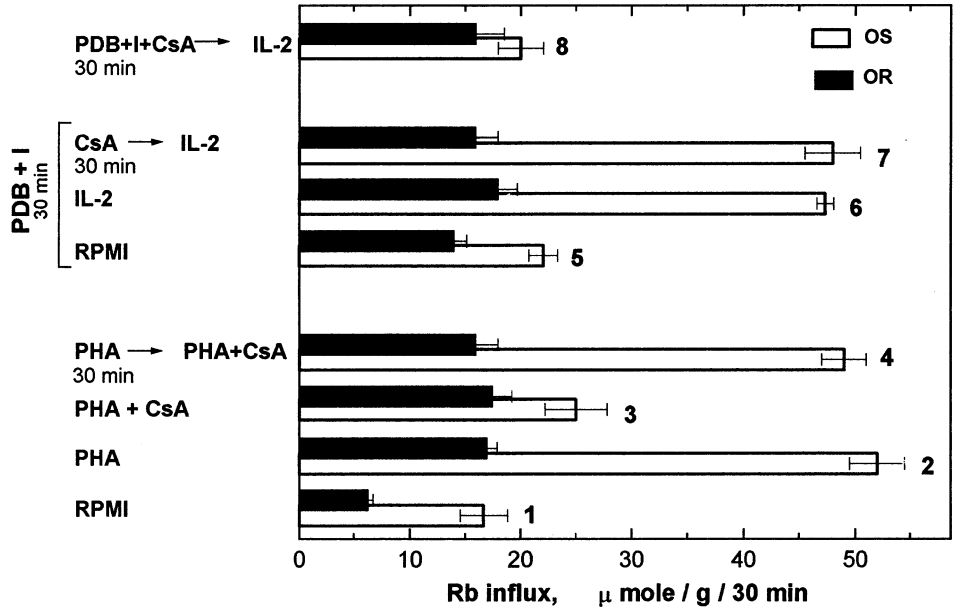

Fig. 3. Proliferation-related long-term enhancement of the Na/K pump is inhibited by CsA during induction of the competence in HBLs. The pump activity was determined as the ouabain-sensitive Rb influx (OS) at 48 h of HBL activation. OR, ouabain-resistant Rb influx. The competence was induced with submitogenic PHA, 2 μg/ml (3) or with phorbol ester, PDB, and ionomycin, I, (5–7) with (8) or without (4–7) CsA. The cell cycle transition was provided by mitogenic PHA, 20 μg/ml (4), or IL-2 (6–8). One representative experiment from seven. Each data point is the mean for triplicate cultures of lymphocytes from one donor.

Table 1
Effect of CsA on the K content and proliferation of HBLs rendered competent by submitogenic PHA (a) or PDB+ionomycin (b) and then stimulated by a mitogenic concentration of PHA or IL-2

	Initial incubation	Second incubation, 48 h	K_{in}, μmol/g, 48 h	Incorporations, $\times 10^3$, counts/mg/30 min, 48 h		
				Thymidine	Leucine	Uridine
	Resting HBLs	RPMI	600 ± 35	0.8 ± 0.1	3.1 ± 0.7	1.3 ± 0.3
(a)						
1		PHA (20)	760 ± 54	267.2 ± 31	14.8 ± 1.5	5.5 ± 0.7
2		PHA (20)+CsA	548 ± 41	88.4 ± 5.3	6.1 ± 0.9	2.1 ± 0.3
3	PHA (2)	PHA (20)+CsA	735 ± 15	179.5 ± 21	8.1 ± 0.6	4.1 ± 0.2
4	PHA (2)+CsA	PHA (20)	575 ± 37	12 ± 1.7	2.4 ± 0.3	1.0 ± 0.2
(b)						
1	PDB+I	RPMI	570 ± 23	42.3 ± 0.8	3.4 ± 0.2	1.5 ± 0.2
2	PDB+I	IL-2	735 ± 48	174.2 ± 27	10.2 ± 0.6	4.7 ± 0.3
3	(PDB+I)+CsA	IL-2+CsA	550 ± 38	31.2 ± 1.3	3.2 ± 0.5	1.6 ± 0.1
4	(PDB+I) → +CsA	IL-2	715 ± 21	157.1 ± 24	8.1 ± 0.6	4.1 ± 0.3

The competence (initial incubation) was induced by submitogenic PHA for 1 h (a) or in the presence of PDB and ionomycin (PDB+I) for 30 min with or without CsA (1 μg/ml) or CsA was added after PDB+I (b). To induce progression (second incubation), cells were incubated in the presence of mitogenic PHA (a) or washed by RPMI and then incubated with IL-2 (200 U/ml) or with IL-2+CsA (b). The numbers in parentheses indicate the concentration of PHA, μg/ml. All the data are obtained on lymphocytes from one donor and each value is the mean of three independent cultures. The results are representative of one of five similar experiments.

Rb influx as well as DNA synthesis and a growth-associated increase in K_{in} (Table 1).

All these results indicate that in HBL, (1) a long-term enhancement of the Na/K pump during the $G_0/G_1/S$ transition is related to a CsA-sensitive pathway of mitogen signalling and (2) the T-cell growth factor, IL-2, is involved in the functional expression of the pump in the course of the pre-replicative phase.

4. Discussion

The effect of growth factors is mediated by transcriptional regulation of a set of genes which shifts the resting cell towards the division cycle. In addition, to realize the growth-promoting signals into a complicated cellular response, many events at the physiological level should occur. Among these, proliferation-related events are the membrane transport processes including an enhanced movement of essential nutrients and ions. It is well-established that most mitogens induce rapid changes in the activity of basic ion transporters, such as Na/H exchange, Na/K pump and Na/K/Cl co-transporter. These transient modulations of ion-transporting systems are mediated by substrate concentrations and the second messengers and are directly involved in the initial mitogen signalling at the cell membrane level.

In recent years, we have discovered a sustained increase in K influxes via the Na/K pump during the later transition of resting cells to proliferation [7,18]. In mitogen-induced cells, the long-term control of ion transport is likely to be mediated by regulating the number of pumps expressed in the plasma membrane. Indeed, our previous study has revealed an increase in the level of α1 and β1 Na,K-ATPase mRNA at 4–8 h of PHA activation of HBLs [20]. Recent studies have also demonstrated changes in the level of Na,K-ATPase mRNA during hepatocyte proliferation and lymphocyte differentiation [21,22]. However, there is a gap of understanding between the transcriptional underlying mechanisms and the whole functional response of the cell to mitogen. The most important aspects of the problem of the ion participation in mitogeneses are (1) how mechanisms controlling the cell cycle-dependent expression of transport proteins might be linked to the multiple signalling pathways which are responsible for the cell transition from the resting state to proliferation and (2) what is the functional meaning of the sustained enhancement of ion fluxes during the cell cycle transition. The present study shows for the first time that in mitogen-activated HBLs, the sustained increase in pump-mediated fluxes is eliminated by anti-proliferative doses of CsA. This suggests a close relationship between the enhanced functional activity of the ion pump during cell cycle progression and the early expression of the T-cell growth factor IL-2 and, perhaps, IL-2R in HBLs.

In conclusion, in most animal cells, the Na/K pump controls (directly or indirectly) many essential functions, such as the cell volume, intracellular pH, free calcium concentration, membrane potential and transport of nutrients. In different types of cells, including HBLs, the action of mitogens is accompanied by a sustained increase in pump-mediated fluxes, this increase being temporally associated with the growth phase of a response [23]. In addition, the transformed cells possess higher ion fluxes as compared to the resting or differentiated cells [4,23]. Being responsible for the cellular ion and osmotic homeostasis, the elevated pump should be involved in genesis of the growth response during the mitogen-induced cell transition towards a new steady state level, which is peculiar for cycling cells. In this aspect, the Na/K pump which belongs to housekeeping cellular factors might be considered as an essential element of the cell cycle regulation at the physiological level.

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