Early and Delayed Changes in Potassium Transport During the Initiation of Cell Proliferation in CHO Culture

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Abstract. Stimulation by serum of cell proliferation in G1-arrested culture of Chinese hamster ovary cells CHO-K1 was accompanied by an early (during the first minutes) and delayed (2—10 h) activation of Na^+,K^+-ATPase and an increase in cell K^+ content from 0.5—0.6 to 0.7—0.8 mmol per gram protein. Isoproterenol acted synergistically with serum in eliciting both early and delayed changes in K^+ transport and in stimulating G1 → S transition. Isoproterenol alone (without serum) induced a transient increase in K^+ influx via Na^+,K^+-ATPase without changing the cell K^+ content or having any mitogenic effect. Theophylline enhanced the serum-induced early activation of Na^+,K^+-ATPase but inhibited both the delayed increase in cell K^+ and the G1 → S transition. Early serum-induced increase in K^+ transport was not affected by cycloheximide, whereas net accumulation of cell K^+ was abolished by the drug. It is concluded that the early and the delayed activation of Na^+,K^+-ATPase induced by mitogens can be dissociated; the early ionic response is related to the primary transduction of membrane signal, whereas the delayed modulation of ion transport via Na^+,K^+-ATPase has another function and is associated with cell growth.

Key words: Potassium transport — Mitogens — Cell proliferation — Isoproterenol — Theophylline

Introduction

The addition of mitogens to quiescent cells is accompanied by a rapid increase in ion fluxes in Na^+,K^+-ATPase, amiloride-sensitive Na^+-H^+ exchange, furosemide-sensitive $Na^+/K^+/Cl^-$ symport and Ca^{2+} fluxes (for recent reviews see Moolenaar 1986; Rozengurt 1986; Vereninov and Marakhova 1986). Similar dose-response relationships have been obtained for early ion changes and initiation of DNA synthesis for various cell lines and during the action of various mitogens; on a number of occasions blocking of the ion transporters

was reported to abolish the mitogenic effect of growth factors. These data suggest that the activation of ion fluxes across the plasma membrane is essential for subsequent proliferation.

On the other hand, there have been reports suggesting that mitogen-induced ion changes are not necessarily required for DNA synthesis to be initiated in a quiescent cell. Under certain conditions the transition of arrested cell cultures to proliferation was not accompanied by the activation of Na^+,K^+-ATPase (Frantz et al. 1981; Sanui and Rubin 1982). Growth factors differ in their ability to raise the Ca^{2+} concentration in the cytoplasm (Frantz 1985; Lopez-Rivas et al. 1987). Some growth factors which induce changes in ion transport may not necessarily initiate mitogenic response (Yarden et al. 1982; Cassel et al. 1984). Apparently, stimulus-induced changes in ion transport may be of different origin and different functional meaning, and, moreover, the lack of mitogenic response in the presence of early ion changes can bear evidence that the cell has not received a complete set of signals and that some other important requirements have not been met.

In studying serum-induced stimulation of proliferation in cultures of Chinese hamster ovary cells CHO-K1, the early activation of Na^+,K^+-ATPase was found to be followed by increases in K^+ influx and cell K^+ content at later stages of G1 → S transition (Marakhova et al. 1986). Delayed changes in K^+ transport in serum stimulated cell cultures were reported also by other authors (Tupper et al. 1977; Frantz et al. 1981), but the relations of these changes to both early activation of Na^+/K^+ pump and mitogenic response have not been studied. The present investigations were designed to compare early and delayed changes in K^+ transport with the initiation of DNA synthesis in CHO cultures induced by serum in combination with agents known to enhance or to diminish the mitogenic effect of serum.

Materials and Methods

CHO-K1-773 cells (Yefimova and Ignatova 1984) were plated out on 50 mm dishes (Anumbra) at a density of 1×10^5 cells per dish containing 5 ml Eagle's medium (MEM) supplemented with 10% calf serum. After 3 days the cultures were confluent to 80–90% and were mostly (up to 90%) arrested in G1 phase of the cell cycle. The G1-arrested cultures were stimulated by completely replacing the depleted medium with 5 ml of fresh Eagle's medium supplemented with 10% calf serum.

Total cell K^+ and Na^+, and K^+ influx were measured by flame spectrophotometry as described elsewhere (Vereninov et al. 1982). To evaluate K^+ influx, Rb^+ was used as an analog of K^+. At various points over the course of the experiment RbCl (final concentration 2.5 mmol/l) was added to the culture medium and the Na^+,K^+-ATPase-mediated K^+ influx was measured as ouabain-inhibitable Rb^+ uptake. The cultures with RbCl and with or without ouabain (1 mmol/l) were incubated for 20 min at 37°C in an atmosphere of humidified CO_2 (5% v/v). Rb^+ uptake was

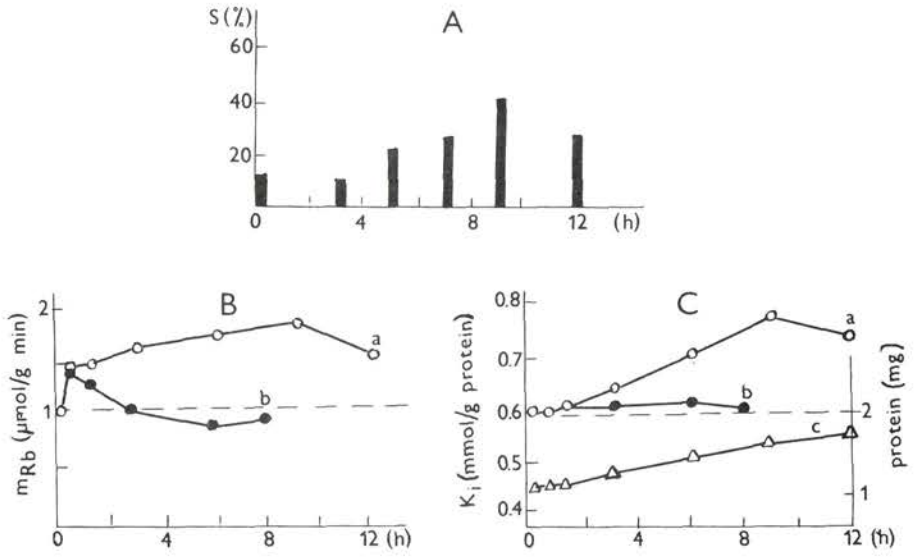

Fig. 1. S phase (*A*), ouabain-inhibitable Rb^+ influx (*B*), cell K^+ (*C*, circles), protein content (*C*, triangles) in CHO-K1 cultures following stimulation with serum (*a*) or serum in the presence of cycloheximide (*b*). G1-arrested CHO-K1 cultures were stimulated to proliferate with fresh calf serum (10 %). At various points the cells were incubated with RbCl (2.5 mmol/l) and ouabain-inhibitable Rb^+ influx was measured after 20 min (see Materials and Methods: 1 mmol/l ouabain was added before RbCl in half of the dishes). Cycloheximide (10 μg/ml) was added before serum. Each cation determination was done in triplicate. Parallel cultures were taken for cytofluorometric analysis. The broken line indicates Rb^+ influx (*B*) and K^+ content (*C*) in unstimulated culture.

stopped by washing the cell monolayer 4 times with 85 mmol/l $MgCl_2$, 5 mmol/l TrisHCl, pH 7.2 at 4 °C and the cells were lyzed with distilled water (2 ml per dish). The samples were analyzed for K^+, Na^+, Rb^+ using a Perkin Elmer AA 306 flame photometer, and for protein content by the Lowry procedure. The cell cation content was related to protein content.

The distribution of the cell cycle phase for CHO cultures, both arrested and stimulated, was assessed using the flow cytometric technique.

Results

Effects of serum on cell proliferation, Rb^+ uptake and cell K^+ content

When the depleted culture medium in G1-arrested CHO cultures was substituted for fresh Eagle's medium supplemented with 10 % calf serum, the cells entered the cell cycle and started within 5—6 h synthesizing DNA. In serum-

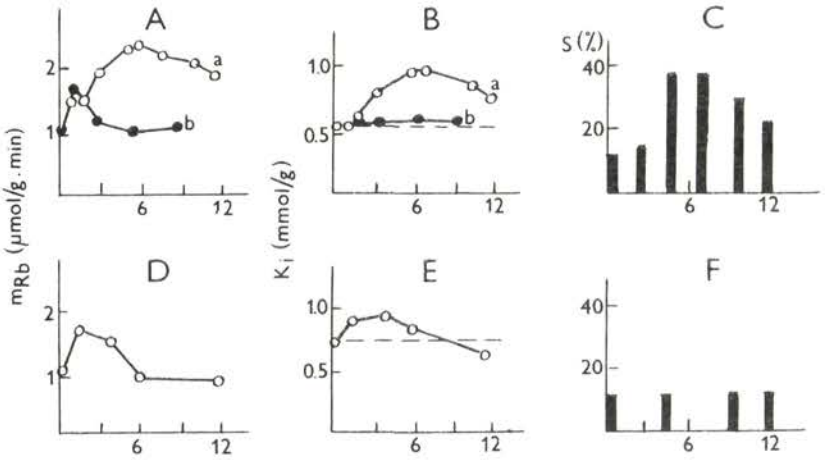

Fig. 2. Ouabain-inhibitable Rb^+ influx (*A*, *D*), K^+ content (*B*, *E*) and S phase (*C*, *F*) in CHO-K1 cultures following stimulation with serum and isoproterenol (*a*), isoproterenol alone (*b*), or serum in the presence of theophylline (*D*—*F*). Serum — 10 %, isoproterenol — 10 μg/ml, theophylline — 2 mmol/l. For experimental conditions see legend to Fig. 1.

stimulated CHO cultures maximum S phase cells (40—50 %) occurred after 8—9 h (Fig. 1*A*).

G1 → S transition in CHO cultures was associated with an immediate (during the first 20 min) and a delayed increase in Rb^+ influx, sustained for 7—9 h (Fig. 1*B*). In contrast, CHO cultures transferred to fresh Eagle's medium without serum showed a constant Rb^+ influx. Serum markedly stimulated the ouabain-inhibitable component of Rb^+ influx mediated by Na^+,K^+-ATPase.

After 2—3 h following serum addition elevated levels of cell K^+ were evident (Fig. 1*C*). The elevation in cell K^+ usually preceded the entry of S phase and parallelled an increase in the cell protein content (Fig. 1*C*). In serum-stimulated CHO cultures the total cell protein increased 1.7—2.0 times over an 8 h period. Interestingly, the increase in cell K^+ content exeeded that in protein content. Consequently, cell K^+ gradually increased between 2 and 9 h from 0.5—0.6 to 0.7—0.8 mmol per gram protein.

Effects of isoproterenol on serum-induced changes in K^+ transport and initiation of DNA synthesis

The mitogenic activity of serum may be enhanced or reduced by hormones or agents the effects of which are mediated by messengers. In CHO cultures the

Table 1. Ouabain-inhibitable Rb^+ influx (m^{Rb}), cell K^+ content (K_i), Na^+ content (Na_i) and S phase (S) in CHO-K 1 cultures stimulated by serum (10 %), isoproterenol (10 g/ml), theophylline (2 mmol/l), propranolol (10^{-6}—10^{-5} mol/l) and their combinations

Additions	m^{Rb} μmol/g min		K_i mmol/g		Na_i protein	S %
	0.5 h	6 h	0.5 h	6 h	0.6 h	8 h
No additions	20	20	600	600	195	10
Serum	33	48	630	740	198	34
Isoproterenol	28	18	597	610	210	10
Propranolol 10^{-6} mol/l	22	21	607	610	220	11
Propranolol 10^{-5} mol/l	20	21	540	500	310	10
Isoproterenol + propranolol 10^{-6} mol/l	19	19	600	590	200	11
Theophylline	28	19	600	590	200	10
Serum + isoproterenol	39	65	610	788	200	47
Serum + isoproterenol + propranol 10^{-6} mol/l	29	45	625	765	270	38
Serum + theophylline	38	20	610	590	187	10

G1 → S transition occurred earlier when the depleted culture medium had been substituted for fresh Eagle's medium containing serum in combination with isoproterenol, a known β-agonist. In the presence of isoproterenol (10 μg/ml) the maximum of S phase cells was observed as early as 5—6 h following medium replacement (Fig. 2*C*).

Isoproterenol enhanced the serum-induced increase in both early Rb^+ uptake and delayed rise in cell K^+ (Fig. 2*A*, 2*B*). When added without serum, isoproterenol also increased ouabain-inhibitable Rb^+ influx (Fig. 2*A*). However in the presence of isoproterenol the change in Rb^+ influx was transient and cell K^+ remained unchanged (Fig. 2*B*). No G1 → S transition could be detected in CHO cultures treated with isoproterenol alone.

The stimulation of Na^+,K^+-ATPase as well as the comitogenic effect of isoproterenol is mediated by β-receptor events, presumably by a rise in the intracellular cAMP content. In our experiments with CHO cells propranolol (10^{-6} mol/l), a β-antagonist, prevented isoproterenol from increasing Rb^+ uptake and accelerating serum-induced G1 → S transition (Table 1).

It is noteworthy that propranolol alone (without isoproterenol pretreatment) had no effects on serum-induced ouabain-inhibitable Rb^+ uptake, cell K^+ content and/or the rate G1 → S progression. At higher concentrations (10^{-5} mol/l) propranolol raised, within 20—30 min, cell Na^+ approximately

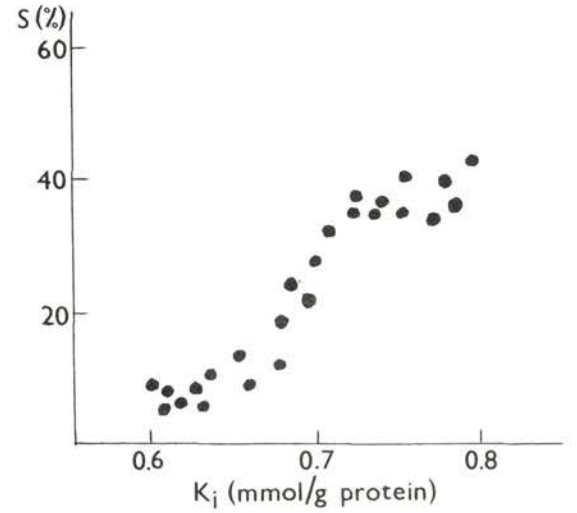

Fig. 3. The relationship between S phase cells and K^+ content in CHO-K1 cultures stimulated by serum or serum in combination with isoproterenol.

2-fold and decreased cell K^+ by 10—15 % (Table 1). These changes in the cation contents may be due to accelerated Na^+ influx and K^+ efflux in propranolol-treated cells and are clearly distinguishable from the β-receptor blocking effect. It is suggested that the effect of propranolol on passive ion fluxes across plasma membrane is related to its local anesthetic properties and that it involves a perturbation in membrane-bound Ca^{2+} (Sarkadi and Gardos 1985).

From a series of experiments with serum- and (serum + isoproterenol) -stimulated CHO cultures a distinct correlation could be derived between the delayed stimulation of K^+ transport and the rate of G1 → S progression (Fig. 3).

Effects of theophylline on serum-induced changes in K^+ transport and initiation of DNA synthesis

As shown in Fig. 1*D*, theophylline, which is phosphodiesterase inhibitor (and thus increases cAMP levels similarly as isoproterenol) enhanced the early stimulation of Na^+,K^+-ATPase, as suggested by an increased ouabain-inhibitable Rb^+ uptake within the first hour of culture stimulation. In contrast to isoproterenol action, theophylline could maintain increased Rb^+ influx in serum-stimulated cultures but over 4 h, when a decline was observable. Theophylline also prevented the elevation of cell K^+ at later stages of G1 → S transition and inhibited the serum-induced reinitiation of DNA synthesis in CHO cultures. The difference between isoproterenol and theophylline in their effects on cell

proliferation appears to be due to the time-course of intracellular cAMP content: a transient rise in cell cAMP, induced by isoproterenol in early G1, acts synergistically with serum in the initiation of DNA synthesis, whereas a prolonged increase in intracellular cAMP levels (by theophylline) blocks G1 → S transition in CHO cultures (Ganelina et al. 1984). These results are in agreement with previous observation on 3T3 cells, which indicated that theophylline inhibits progression through the cell cycle in G1 phase (Tupper et al. 1977). Thus, theophylline disrupts the early activation of Na^+,K^+-ATPase and the delayed K^+ accumulation as well as the S phase of serum-stimulated CHO cultures.

Effect of cycloheximide on serum-induced changes in K^+ transport

To test whether Na^+,K^+-ATPase activation and K^+ accumulation in serum-stimulated cell culture depends on protein synthesis, we examined the effects of cycloheximide on Rb^+ uptake and intracellular cation contents. In the presence of cycloheximide (10 μg/ml) serum-stimulated CHO cultures did not initiate DNA synthesis and failed to increase cell protein. The rapid early increase in Rb^+ uptake induced by serum was not affected by cycloheximide (Fig. 1*B*). In contrast, the delayed increase in Rb^+ were completely abolished by the drug. It should be noted that cycloheximide, when added to unstimulated CHO cultures for 6—8 h, had no effect on Rb^+ influx and/or K^+ and Na^+ contents. Obviously, the mechanisms underlying the early and delayed activation of Na^+,K^+-ATPase are different.

Discussion

The present study shows that serum-induced stimulation of G1 → S transition in arrested CHO cultures is associated with both immediate (early) and prolonged (delayed) increase in $Rb^+(K^+)$ influx due to the elevation of ouabain-inhibitable transport by Na^+,K^+-ATPase. The sustained delayed stimulation of Na^+,K^+-ATPase and the increase in cell K^+ content per gram protein is typical of successful G1 → S transition in serum-stimulated cell cultures.

It is vell established now that various mitogens which initiate DNA synthesis in quiescent cells rapidly stimulate Na^+,K^+-ATPase. In addition certain hormone-induced physiological responses are also associated with the increase in K^+ influx via Na^+,K^+-ATPase (Ihlenfeldt 1981; Moore 1983; Lynch et al. 1987). It is assumed that a rapid increase in K^+ influx compensates for the elevated K^+ efflux observed in many cells after hormone-receptor interaction. Thus, early ion flux changes might represent a common pathway of cellular response to an exogenous signal regardless of the nature of the signal factor.

In this study attention has been focused on delayed ion changes in mitogen-stimulated cells. Evidence has been presented for the delayed (but not the early) sustained activation of Na^+/K^+ pump and the increase in cell K^+ being in some way essential for the resting cell to enter the S phase. In fact, stimuli which induce only early increase in K^+ influx (isoproterenol, theophylline, serum plus theophylline) fail to initiate DNA synthesis in arrested CHO cultures. Serum or serum in combination with isoproterenol induce both early and delayed activation of Na^+,K^+-ATPase and raise cell K^+. These agents are also capable of initiating DNA synthesis. A strict correlation was found between the rate of G1 → S progression and the increase in cell K^+ in serum-stimulated CHO culture.

Both the early and the delayed activation of Na^+,K^+-ATPase by mitogens can be disrupted and seem to be regulated by different mechanism. This assumption is favoured by the evidence that the early enhancement of ouabain-inhibitable K^+ transport is not affected by cycloheximide, whereas the delayed modulation of Na^+,K^+-ATPase-mediated K^+ transport is suppressed by the drug. Long-term regulation of Na^+,K^+-ATPase during G1 → S progression is likely to be associated with protein synthesis and might be related to gene expression as opposed to the early activation of Na^+/K^+ pump, which is supposed to be due to an increased Na^+ concentration (Rozengurt 1980); alternatively, the early activation might be mediated by messengers (allosteric regulation of the ion-transporting complex).

Of particular interest in this study is the increase in cell K^+ per gram protein in CHO cultures stimulated to proliferate. It has been shown that the initiation of DNA synthesis in cultures of 3T3 cells is dependent on cell K^+ content: DNA synthesis occurs only when the intracellular K^+ increases above a certain threshold, which is around 0.56 mmol per gram protein (Lopez-Rivas et al. 1982). The inhibition of G1 → S transition when cell K^+ decreases below the threshold has been assumed to be the result of protein synthesis control (Ledbetter and Lubin 1977). The question arises what is the functional meaning of the increase in cell K^+ above the threshold during G1 → S transition?

It is known that a quiescent cell entering the cycle is gaining in mass and volume during G1 phase. Under physiological conditions DNA synthesis is always preceded by a growth in cell size (Baserga 1984). A comparison of the patterns of G1 → S transition and delayed changes in K^+ transport reveals parallelism of the rising cell K^+ content and protein mass. In other words, delayed changes in K^+ transport are likely to be associated with cell growth.

K^+ is the major intracellular osmotic agent and an increase in its content per cell or per protein could lead to a rise in water content per cell or per gram protein. The entering water will "dilute" the cell K^+. For this reason variations in cell K^+ concentration (i.e. K^+ content per cell water) are always much smaller

than changes in K^+ content per cell or per gram protein. In fact, spare data available show that no significant changes in cell K^+ concentration occur during initiation of mitogenic response (Frantz et al. 1981; Amsler et al. 1985). The principal conclusion drawn is that any change in cell K^+ content per gram protein should be parallelled by a corresponding change in water content per gram protein. It might be assumed that the functional meaning of changes in cell K^+ content in cells entering the cycle is not so much in the rise in cell K^+ concentration, but rather in the rise in water content per gram protein.

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