Regulation of IGF-1-dependent cyclin D1 and E expression by hEag1 channels in MCF-7 cells: The critical role of hEag1 channels in G1 phase progression

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ABSTRACT

Insulin-like Growth Factor-1 (IGF-1) plays a key role in breast cancer development and cell cycle regulation. It has been demonstrated that IGF-1 stimulates cyclin expression, thus regulating the G1 to S phase transition of the cell cycle. Potassium (K^+) channels are involved in the G1 phase progression of the cell cycle induced by growth factors. However, mechanisms that allow growth factors to cooperate with K^+ channels in order to modulate the G1 phase progression and cyclin expression remain unknown. Here, we focused on hEag1 K^+ channels which are over-expressed in breast cancer and are involved in the G1 phase progression of breast cancer cells (MCF-7). As expected, IGF-1 increased cyclin D1 and E expression of MCF-7 cells in a cyclic manner, whereas the increase of CDK4 and 2 levels was sustained. IGF-1 stimulated p21$^{WAF1/Cip1}$ expression with a kinetic similar to that of cyclin D1, however p27^{Kip1} expression was insensitive to IGF-1. Interestingly, astemizole, a blocker of hEag1 channels, but not E4031, a blocker of HERG channels, inhibited the expression of both cyclins after 6–8 h of co-stimulation with IGF-1. However, astemizole failed to modulate CDK4, CDK2, p21$^{WAF1/Cip1}$ and p27^{Kip1} expression. The down-regulation of hEag1 by siRNA provoked a decrease in cyclin expression. This study is the first to demonstrate that K^+ channels such as hEag1 are directly involved in the IGF-1-induced up-regulation of cyclin D1 and E expression in MCF-7 cells. By identifying more specifically the temporal position of the arrest site induced by the inhibition of hEag1 channels, we confirmed that hEag1 activity is predominantly upstream of the arrest site induced by serum-deprivation, prior to the up-regulation of both cyclins D1 and E.

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1. Introduction

Ion channels, including potassium (K^+) channels, are important in the cell cycle and thus, in cell proliferation. They are found in a variety of cancer cells [1–3].

It has been shown that Eag-1 K^+ channels, a member of the voltage-activated K^+ channel superfamily, play a role in controlling the proliferation and transformation of epithelial cells [1,4]. They might be considered as potential markers for molecular staging and targets for the development of anticancer therapeutic strategies [5]. Increased expression of Eag-1 channels is found in several cancer types including breast cancer [6,7]. The inhibition of Eag-1 channel activity suppresses the proliferation of breast cancer cells and accumulates cells in G1 phase of the cell cycle [8,9]. Although no direct evidence of the mechanism has been provided, consequences of the inhibition of K^+ channels suggest that these channels may be involved in the control of the cell cycle machinery, particularly the regulation of cyclin-dependent kinase inhibitors (CDKI) and/or cyclin expression [10,11].

The progression of cells through the cell cycle is regulated by different cyclin/CDK complexes. These molecules form the regulatory (cyclins) and catalytic (CDK, Cyclin-Dependent Kinase) subunits of cell cycle-regulated kinase. After growth-factor stimulation, the first cyclins to appear when quiescent cells enter the cell cycle are the D-type cyclins (D1, D2 and D3). D-type cyclins bind, and thereby activate, CDK4 and CDK6. Activation of the cyclin D/CDK4–6 complex promotes G1 phase progression. The sequential activation of the cyclin E/CDK2 complex and hyperphosphorylation of pRb are necessary for the release of the E2F (E2 promoter-specific factor) transcription factor leading to DNA synthesis. Cyclins D and E are thus the main activators of the G1 to S phase transition and therefore for the entry into S phase. The activity of the cyclin/CDK complex can be inhibited by cyclin-dependent kinase

inhibitors (CDKI), which are activated to prevent disorder in the cell cycle machinery. Indeed, p21[WAF1/Cip1] and p27[kip1] bind to cyclin/CDK complexes and regulate the G1–S transition by inhibition of the complex activity [12].

In MCF-7 cells, cyclin D1 is the essential D-type cyclin [13,14] and its role in the cell cycle progression is well established [15]. Both cyclins D1 and E are overexpressed in several tumors including breast cancer. In fact, cyclin D1 overexpression was reported in 30–60% of primary ductal breast adenocarcinomas [16,17]. Different cyclin E isoforms are overexpressed in 40% of breast cancers and are associated with the tumor stage, grade and Ki67 proliferative index [18,19].

IGF-1 is a strong mitogen factor for a wide variety of cancer cell lines, including sarcoma, leukemia, prostate, breast, lung, colon, stomach, esophagus, liver, pancreas, kidney, thyroid, brain, ovary, and uterus cancers [20,21]. IGF-1 has been shown to regulate the expression of cyclin, CDK proteins and Rb protein phosphorylation on breast cancer cell lines including MCF-7 cells. In serum-deprived MCF-7 cells, stimulation by IGF-1, through the PI3K pathway, enhances the expression of cyclin D1 and cyclin E, hyperphosphorylation of pRb and subsequently, cell proliferation [22].

We previously demonstrated that hEag1 K$^+$ channels are involved in the control of the cell cycle progression and proliferation of MCF-7 cells [8,9]. We also demonstrated that IGF-1 increased both the activity and expression of hEag1 channels through a PI3-Kinase/Akt dependent pathway [23]. Moreover, pharmacologically or more specifically, the inhibition of hEag1 K$^+$ channels by siRNA decreased the mitogenic effect of IGF-1 on MCF-7 cells.

In this study, we more thoroughly investigated the involvement of hEag1 K$^+$ channels in the G1 phase progression and determined whether or not these channels could be implicated in the IGF-1 regulation of cyclin (D1, E), CDK (4, 2), or CDKI (p21[WAF1/Cip1], p27[kip1]) expression. This study is the first to demonstrate that the hEag1 K$^+$ channel is a key actor of the IGF-1 pathway which modulates cyclin D1 and E expression, leading to G1 progression and S phase transition and therefore, cell proliferation.

2. Material and methods

2.1. Cell culture

The MCF-7 breast cancer cell line was obtained from the American Type Culture Collection. MCF-7 cells were grown in Eagle's Minimum Essential Medium (EMEM; Gibco) supplemented with 5% fetal bovine serum (FBS), L-glutamine (2 mM), and gentamicin (50 μg/ml) (Gibco). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO$_2$.

To obtain cells accumulated in G1 phase, cells were incubated in serum- and phenol red-free EMEM medium for 24 h [8,9]. This environment was used as the control condition in all our experiments to study the effects of IGF-1. Cells were then stimulated with IGF-1 (20 ng/ml) to enter the cell cycle.

2.2. Temporal position of the synchronized site in G1 phase

To determine the temporal position of the arrest site in G1 phase induced by astemizole and lovastatin, MCF-7 cells were grown in EMEM medium with 5% FBS for 48 h and then treated with astemizole (5 μM) (Sigma, France) or lovastatin (20 μM) (Sigma, France) for 48 h and 39 h respectively. After treatment, the medium was replaced with drug free-EMEM medium to resume cell progression in the cell cycle. 2 mM of mevalonic acid (Sigma, France) was added in the medium to reverse the effect of lovastatin. After different times of removal, the cells were then harvested and the cell distribution in the cell cycle was measured by flow cytometry.

2.3. Flow cytometry

Cell cycle analysis was performed by measuring cellular DNA content by flow cytometry. Cells were collected by trypsinization, resuspended in 300 μl PBS-EDTA (5 mM) and fixed with 700 μl absolute ethanol. After fixation, cells were pelleted by centrifugation, resuspended in PBS-EDTA (5 mM), treated with ribonuclease at a final concentration of 10 μg/ml (Sigma, France) for 30 min and stained with propidium iodide (Sigma, France) at a final concentration of 50 μg/ml. To assess cell cycle distribution patterns (G0/G1, S and G2/M phases), the stained samples were measured using a flow cytometer (Elite Beckman/Coulter, USA).

2.4. Western blotting analysis

Treated cells were washed twice in the phosphate buffered saline (PBS), lysed in Laemmli's buffer (Tris-base 62.5 mM pH 6.8, glycerol 10%, 2-βmercaptoethanol 5%, SDS 2.3% and 0.025% of bromophenol blue) and heated three times to 95–100 °C for 10 min. The lysate was analyzed by immunoblotting. Equal amounts of protein were separated by electrophoresis on SDS-PAGE and electrotransferred to a nitrocellulose membrane. Blots were blocked in 5% nonfat milk for 1 h and then incubated overnight at 4 °C in Tris-buffered saline containing Tween 20 (0.1%) (TBS-T) with a primary antibody directed against either cyclin D1 (DS26, 1:500), p21[Waf1/Cip1] (12D1, 1:500), p27[Kip1] (1:500), phospho-Rb[ser807/811] (ppRb [ser807/811]) (1:500), protein Rb (pRb) (4H1, 1:1000, Cell Signaling Technology), CDK4 (C-22, 1:1250), cyclin E (C-19, 1:1300), CDK2 (M2, 1:2000), or actin (used as an internal standard) (C-11, 1:2000, Santa Cruz Biotechnology). Membranes were washed three times in TBS-T and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After three washes in TBS-T, the immuno-reactive bands were detected using the ECL chemiluminescent system (Amersham-Biotech), visualized with the ChemiDoc XRS system (Biorad) and quantified using the Quantity One software (Biorad).

2.5. siRNA cell transfection

The hEag1 siRNA sequence used was 5′-CUGGACAUGGACCAA-GUGGAC(dTdT)-3′ (positions 1578–1598 on the hEag1a sequence and positions 1659–1679 on the hEag1b sequence, accession numbers AF078741 and AF078742, respectively) as described by Weber et al. [24]. Control experiments were performed by transfecting a functional non-coding siRNA as a negative control (Dharmacon Research Inc., USA). 2×10^6 MCF-7 cells were transfected using a Nucleofector device and the corresponding kit (Amaxa Inc., Germany). Transfection protocols were performed following the manufacturer's instructions (program E-014; solution V). Using the nucleofection technique, Gresch et al. [25] reported that the nucleofection by 1 μM siRNA is efficient, optimal and without non-specific effects up to 5 μM. Based on these results, we chose to use hEag1 siRNA (si hEag1) at 2 μg (equivalent to 1.3 μM). Immediately after transfection, cells were cultured in EMEM medium with 5% FBS. After treatment, cells were then prepared for immunoblotting studies.

2.6. Drugs and plasmids

For all experiments, MCF-7 cells were activated with recombinant human IGF-1 (Promocell GmbH). Astemizole (AST) (Sigma, France) was used as a blocker of hEag1 channels and E-4031 (Sigma, France) as a specific blocker of HERG channels. The final concentration of DMSO was ≤1/1000.

2.7. Statistical analysis

Data are presented as mean ± SD. Averages of the kinetics were tested with a one-way ANOVA followed by the Dunnett post-test (control was the 0 h point). Differences between the values were considered significant when $p < 0.05$. The p values of <0.05, <0.01 and <0.001 are represented as *, ** and *** respectively.

3. Results

3.1. Effect of IGF-1 on both the cell cycle phase distribution of MCF-7 cells and cell cycle protein expression

MCF-7 cells, in serum- and phenol red-deprived medium for 24 h, were growth-arrested in G1 phase [8]. The addition of IGF-1 induced a cohort of cells to re-enter the cell cycle [26]. In these conditions, we observed the relationship between the cell cycle position and cyclin gene expression induced by IGF-1 in MCF-7 cells. First, we determined the time required for serum- and phenol red-deprived MCF-7 cells to exit from the G1 phase and enter into S phase after IGF-1 stimulation. In order to do this, cells were harvested after IGF-1 treatment at different incubation times for DNA analysis by flow cytometry. Fig. 1 shows that after 8 h of IGF-1 stimulation, the proportion of cells in G1 phase began to decrease, whereas the proportion in S phase simultaneously began to increase. The cell distribution in S phase was clearly modified after 12 h of incubation and became higher after 14 h. These results indicate that the time required for cells to exit from the G1 phase and enter into S phase was between 8 and 10 h after the addition of IGF-1. After 14 h of stimulation, the cell rate was at its maximum in S phase. Next, we studied the expression pattern of the key proteins in G1 and S phase induced by IGF-1 in this time interval. MCF-7 cells were cultured in the same culture condition as above. The levels of these proteins were determined by Western blotting. Our results show that IGF-1 increased the cyclin D1 level with a maximum observed after 4–6 h of stimulation (~two-fold). This effect was

sustained until 10 h and was abolished at 12 h of stimulation (Fig. 2A). The highest expression for cyclin E was obtained at 8–10 h of IGF-1 incubation (~two-fold) as the cells entered in S phase, and then declined after 14 h (Fig. 2B). IGF-1 also enhanced the expression of CDK4 and CDK2 after 4 and 8 h of stimulation, respectively (Fig. 2A and B). As expected, IGF-1 increased cyclin D1 and E expression in a time- and cyclic-dependent manner. On the other hand, the modulation of the catalytic subunits by IGF-1 was sustained over time. Moreover, no modulation of the p27[kip1] protein level by IGF-1 was observed (Fig. 2C). In contrast, IGF-1 stimulation resulted in an increase in the p21[Waf1/Cip1] protein level in the same kinetic as cyclin D1 (Fig. 2C).

3.2. Involvement of hEag1 channels in the up-regulation of cyclin D1 and E expression by IGF-1

To determine if hEag1 channels are involved in the up-regulation of G1 and S phase key proteins induced by IGF-1, we treated serum- and phenol red-deprived MCF-7 cells with 20 ng/ml of IGF-1 or pre-incubated 30 min with astemizole (5 μM) and treated in the presence of both IGF-1 and astemizole. Cells were harvested at the indicated times and the cell cycle proteins levels were analyzed by Western blotting. After 4 h and 6 h of both IGF-1 and astemizole treatment, we observed an increase of cyclins D1 and E, respectively, leading to entry into the cell cycle (Fig. 3A*a–b* and B*a–b*). This was confirmed by a FACs analysis (Supplemented data 1) which showed that the astemizole treatment did not affect the distribution of cells in the cell cycle phases after 24 h. However, we detected a significant decrease in cyclin D1 and cyclin E expression after 6 h and 8 h of astemizole treatment respectively (Fig. 3AB*a–b*). This decrease was maintained until 48 h. Moreover, at 48 h, in the presence of astemizole, the results show that the cyclin D1 protein level was significantly lower than the control condition (serum-deprivation) (Fig. 3A-*a*) and that MCF-7 cells were arrested in G1 phase (supplemented data 1). We also detected that the inhibition of cyclin expression was accompanied by a strong decrease of Rb protein phosphorylation after 48 h of IGF-1 and astemizole treatment, demonstrating the lack of cyclin/CDK complex activity (Fig. 3C). However, astemizole failed to modulate the CDK4, CDK2, p21[Waf1/Cip1] and p27[kip1] protein levels (supplemented data 2). Taken together, our results suggest that the inhibition of hEag1 channels: i) triggered an inhibition of cyclin D1 and E expression leading to their faster turn-over, and ii) induced a decrease of cyclin D1 expression below its level in serum-deprived cells. Therefore, we concluded that the astemizole-induced cell cycle arrest of MCF-7 is managed by the down-regulation of cyclin.

Astemizole has also been known to block HERG potassium channels [27,28]. To test the implication of these channels in the regulation of cyclin expression, we used a specific HERG channel blocker (E-4031). E-4031 at 1 μM is known to specifically block HERG channel activity. We used this inhibitor in the same conditions as described for the astemizole treatment. Our results show that after 8 h of treatment, E-4031 had no effect on the cyclin D1 and E protein levels, whereas in the presence of astemizole, cyclin D1 and E expression was decreased (Fig. 4A). These results suggest that hEag1 channels, but not HERG channels, contributed to the regulation of cyclin expression.

To confirm these results, we used siRNA technology to more specifically down-regulate hEag1 channel expression. MCF-7 cells were thus transfected with hEag1 siRNA using the Nucleofector technology. The effect of hEag1 siRNA (si hEag1) was compared to transfected cells with control siRNA (see the Material and methods section). After transfection, cells were incubated in culture medium for 48 h and then serum- and phenol red-deprived for 24 h. Cells were harvested either after this treatment or after 8 h of IGF-1 stimulation. Fig. 4B shows that transfection with si hEag1 reduced cyclin D1 and E protein levels as compared to the control siRNA, either in the presence or absence of IGF-1. Altogether, our results clearly show that the effect
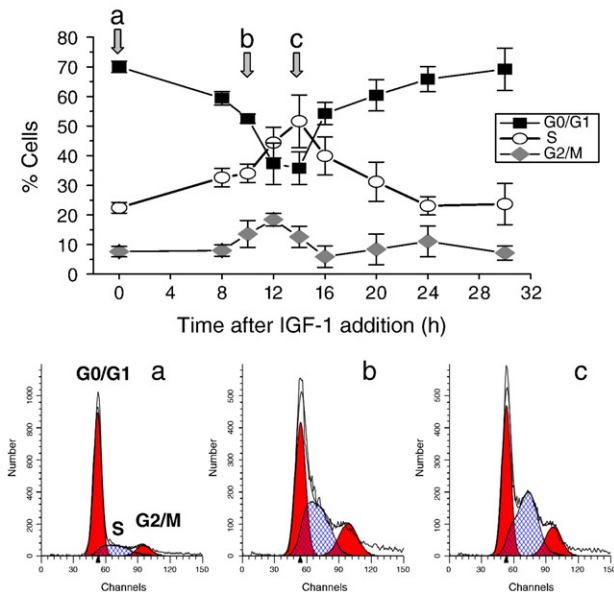

Fig. 1. Time course of IGF-1 effect on the distribution in cell cycle of serum-deprived MCF-7 cells. MCF-7 cells were arrested in G1 phase by serum- and phenol red-deprivation for 24 h (Time = 0). To determine the time required to emerge from G1 phase and enter into S phase by the addition of IGF-1 (20 ng/ml), cells were stimulated with IGF-1 and collected at the indicated times. Cells were then stained with propidium iodide, and flow cytometry was used to determine the percentage of cells in G0/G1, S and G2/M phases at the indicated times. *Top*, each point represents the mean ± SD from three experiments. *Bottom*, representative distribution of cells in the cell cycle at three defined points of the kinetic (a, b and c).
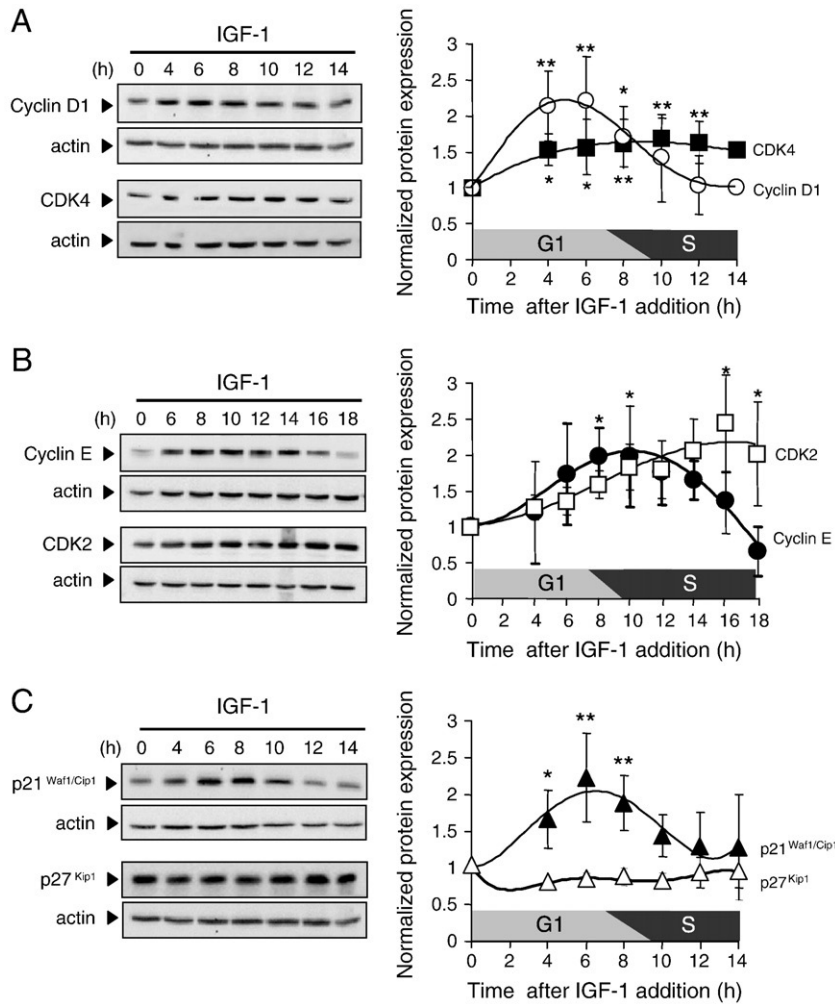
Fig. 2. Effect of IGF-1 on the expression of G1 and S phase key proteins. MCF-7 cells were serum- and phenol red-deprived for 24 h (Time = 0) and then stimulated with IGF-1 (20 ng/ml). Cells were harvested at the indicated times and lysed in Laemmli's buffer. Cell lysates were analyzed by Western blotting using primary antibodies directed against cyclin D1 and CDK4 (A), cyclin E and CDK2 (B), p21$^{Waf1/Cip1}$ and p27^{Kip1} proteins (C). For each protein, a representative immunoblot of three independent experiments is shown (left panel). Protein levels were quantified and normalized to actin. The indicated values are the mean ± SD of three independent experiments (right panel). *p<0.05; **p<0.01.

of hEag1 channel on the cell cycle was mediated by a strong control of cyclin D1 and E expression.

3.3. Determination of the temporal position of the arrest site induced by hEag1 channel inhibition

In order to clarify the involvement of hEag1 channels in G1 phase, we specifically studied the temporal position of the arrest site induced by astemizole in G1 phase using flow cytometry. As astemizole is reversible [29], MCF-7 cells resume their progression through the cell cycle after removal of this drug. We thus estimated the time required for MCF-7 cells to exit the arrest site in G1 phase and enter into S phase. To locate the site of arrest by astemizole, we compared the temporal properties of arrest induced by astemizole with that of arrest induced by either serum- and phenol red-deprivation (Fig. 1) or lovastatin. Lovastatin is well known to block the cells in early G1 phase [30,31]. Lovastatin inhibits 3-hydroxy-3methylglutaryl coenzyme A reductase and its effect is rapidly reversed by the addition of mevalonic acid (2 mM) to the culture medium. MCF-7 cells were thus arrested in G1 phase by lovastatin (20 μM) treatment for 39 h. After removal of lovastatin by the addition of mevalonic acid, we demonstrated that MCF-7 cells exited the lovastatin arrest site and entered into S phase after an 18–20 h lag period. The number of cells in S phase peaked at 24–26 h after drug removal and then began to decrease, in correlation with an

increase in the number of cells in G2/M phase (Fig. 5A). Therefore, we estimated that the duration of G1 phase was approximately 20 h long.

Afterwards, we determined the temporal position of the arrest site induced by astemizole. Cells were treated with astemizole (5 μM) for 48 h. After treatment, the medium was replaced with drug-free EMEM medium. Cell growth was restored and no effect was observed on cell mortality (data not shown). Our results clearly demonstrate that the release of MCF-7 cells from the arrest site by astemizole entered into S phase between 10 and 12 h, and that the number of cells in S phase peaked at 16 h after drug washout (Fig. 5B). When these data are considered together, we showed that after drug removal, lovastatin-treated cells needed 18–20 h to enter into S phase (Fig. 5A), astemizole-treated cells needed 10–12 h (Fig. 5B) and serum-deprived cells needed 8–10 h (Fig. 1). Our results suggest that the arrest site induced by astemizole is upstream of the arrest site induced by serum-deprivation but downstream of the early arrest site induced by lovastatin. We thus confirmed that hEag1 activity occurs in mid-G1 phase, prior to the up-regulation of both cyclins D1 and E.

4. Discussion

IGF-1 is known as an inductor of the cell cycle by inducing the cyclin and CDK expression. Although its biological effect on the cell cycle machinery is well known, the contribution of ionic channels has
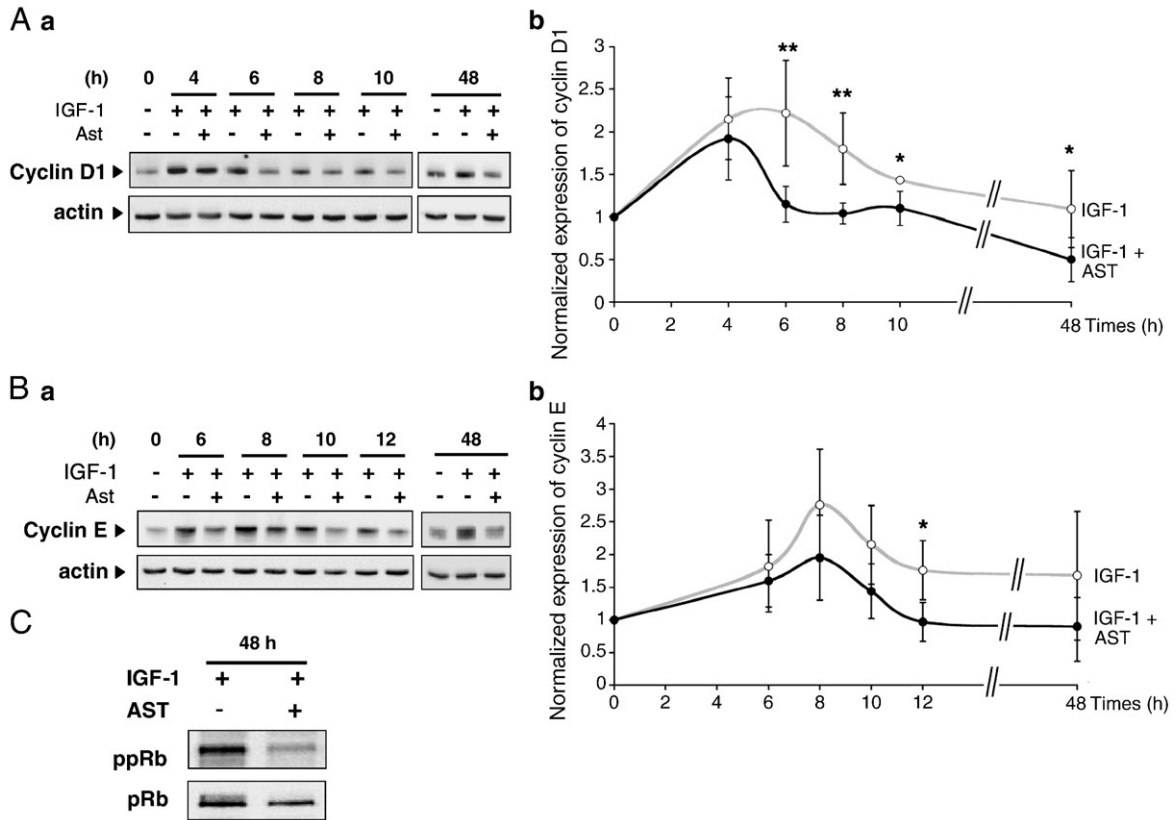
Fig. 3. Astemizole treatment prevented the IGF-I-induced upregulation of cyclin D1 and E protein levels. Serum and phenol red-deprived MCF-7 cells were stimulated with 20 ng/ml of IGF-1 or pre-incubated 30 min with astemizole (5 μM) and treated in the presence of both IGF-1 and astemizole. Cells were harvested at the indicated times and the levels of cyclin D1 (A) and cyclin E (B) were analyzed by Western blotting. a: A representative immunoblot of three experiments is shown. b: Kinetics showing the evolution of the cyclin levels in both conditions. Protein levels were quantified and normalized to actin. The variation in the protein level between the IGF-1 treatment and IGF-1 + astemizole treatment was statistically assessed for each point of the kinetics. The indicated values are the mean ± SD of five independent experiments. *$p < 0.05$; **$p < 0.01$. C: A representative immunoblot of the effect of hEag1 inhibition on phosphorylated Rb protein (ppRb) vs Rb protein (pRb).

not yet been clearly described. The aim of the present study was to elucidate the involvement of a class of K$^+$ channels known to be involved in controlling breast cancer cell cycle progression, and which is regarded as a tumor marker, in the regulation of hEag1 K$^+$ channels.

In MCF-7 cells, IGF-1 treatment increased cyclin D1, cyclin E and p21$^{Waf1/Cip1}$ protein levels in a cyclic-dependent manner but did not modulate p27^{kip1} expression, a typical expression pattern of the proliferation response to diverse mitogenic growth factors. These results are consistent with well known mechanisms describing cell cycle machinery regulation by IGF-1 in MCF-7 cells [22,32,33]. p21$^{Waf1/Cip1}$, originally identified as an inhibitor of the cyclin/CDK complexes, has also been shown to have a role as an adaptor protein that assembles and promotes the kinase activity of cyclin D/CDK4 complexes [34] and thus, is acknowledged as a positive regulator in the cell proliferation induced by IGF-1 in MCF-7 cells [32]. As it is well known that the activity of cyclin D1/CDK4 and cyclin E/CDK2 complexes are optimal during late G1 phase and at the G1/S transition, the time course for the expression of these different proteins correlates with our results on the IGF-1-induced cell redistribution of the cell cycle. Moreover, we show that the CDK4 and CDK2 protein levels were markedly and sustainably increased in response to IGF-1 treatment. The induction of CDK protein levels is not restricted to IGF-1 stimulation because it was also observed after treatment with other potent breast cancer mitogens, such as insulin [35].

In 1996, Wonderlin and Strobl [11] showed that K$^+$ channels are involved in the first events of G1 phase, and thus suggested that their activity should be necessary to the establishment of the events preceding DNA synthesis. The up-regulation of cyclins D1 and E by growth factors is the first event prior to the entry of cells into S phase. In 2001 and 2004, our group proposed hEag1 K$^+$ channels as an initiating actor of G1 phase

[8,9]. It is for this reason that we studied the effect of hEag1 channel inhibition on IGF-1-regulation of cyclin expression.

In the presence of astemizole or siRNA, the amount of cyclin D1 is significantly reduced after treatment. After 48 h of hEag1 channel inhibition, we observed a decrease of cyclin D1 expression, which was accompanied by a strong decrease of Rb phosphorylation and an arrest of the cell cycle in G1 phase. Cyclin D1 is reported as being an important regulator of cell cycle initiation. The crucial role of cyclin D1/CDK4 in G1 phase progression in MCF-7 cells is suggested since the inhibition of cyclin D1 expression by antibodies or siRNA resulted in hypophosphorylation of Rb protein, G1 arrest and cell proliferation inhibition [15,36]. Our results show that hEag1 channel inhibition: (i) induced a significant decrease of an important actor involved in cell cycle initiation, and (ii) produced similar results to cyclin D1 inhibition. Moreover, the effect of hEag1 channel inhibition is accompanied by a decrease of cyclin E expression, the key regulator of the G1/S transition and necessary for the entry of cells into S phase.

Astemizole treatment produced a significant decrease of cyclin D1 after 6 h. The protein level is always the sum of synthesis and degradation. Therefore, it may be hypothesized that either synthesis was slowed down or degradation increased. The half-life time of cyclin has been shown to be quite short, at approximately 45 min [37]. Furthermore, we demonstrated that the decrease of cyclin D1 expression occurred until 48 h of treatment. Therefore, this strong decrease of cyclin D1 after 6 h of astemizole treatment is likely due to the inhibition of cyclin D1 synthesis induced by hEag1 blockage. According to the literature, the first 4 h of cyclin D1 expression is sufficient to trigger cell cycle entry, which is why the cell starts cycling even in the presence of astemizole [35]. This also suggests that the
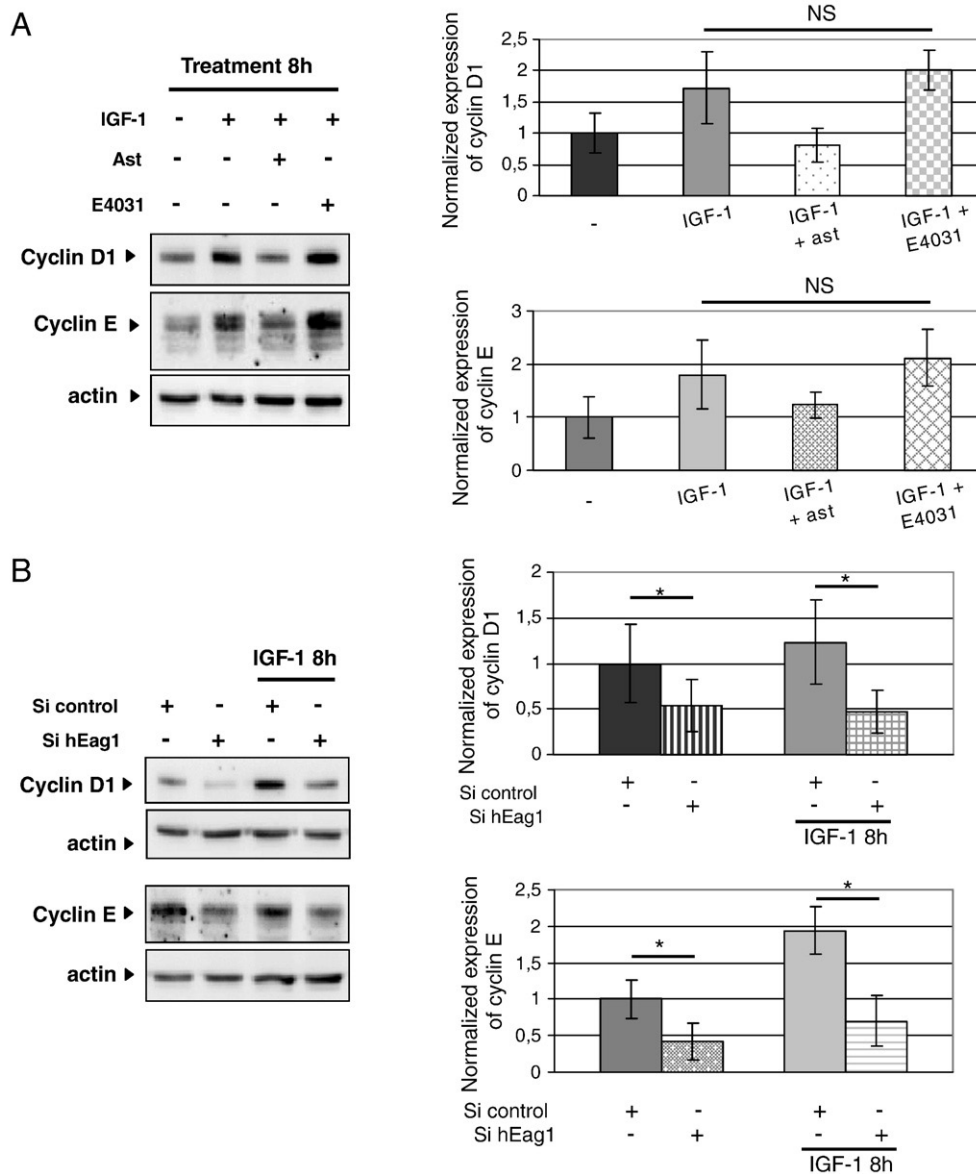
Fig. 4. Involvement of hEag1 channels in cyclin D1 and E expression. (A) After cells were serum- and phenol red-deprived for 24 h, they were then stimulated with 20 ng/ml of IGF-1 or pre-incubated 30 min with either astemizole (5 μM) or E-4031 (1 μM), a specific blocker of HERG channels, and then treated by IGF-1 in the presence of astemizole or E4031 for 8 h. The cyclin D1 and E protein levels were determined by Western blotting. The results shown are representative of the two independent experiments. (B) MCF-7 cells were transfected by a Nucleofector device with either control siRNA (Si control) or hEag1 siRNA (Si hEag1), and then cultured in EMEM medium with 5% FBS. After 48 h, cells were serum- and phenol red-deprived and then stimulated with IGF-1 (20 ng/ml). The cyclin D1 and E protein levels were analyzed after 24 h of serum- and phenol red-deprivation and after 8 h of IGF-1 stimulation. For each experiment, a representative immunoblot of three independent experiments is shown (left panel). Protein levels were quantified and normalized to actin. The indicated values are the mean ± SD of three independent experiments (right panel). NS = not significant result; *$p < 0.05$.

control of cyclin D1 expression by hEag1 channels is quite a slow phenomenon (more than 4 h).

After 48 h of astemizole treatment, we observed that the decrease of cyclin D1 expression is below its level in serum-deprived cells. We thus hypothesized that the hEag1 channel control of the cell cycle would be upstream of the G1 phase synchronization by serum deprivation, since the cyclin D1 protein level is lower in early G1 phase events. By more specifically identifying the temporal position of the arrest site induced by the inhibition of hEag1 channels, we demonstrated that the arrest site induced by astemizole is upstream of the arrest site induced by serum-deprivation but downstream of the early arrest site induced by lovastatin. We thus confirmed that hEag1 activity occurs upstream of the up-regulation of both cyclins D1 and E. Taken together, this study is the first to demonstrate the importance of hEag1 K+ channel activity in the events preceding DNA

synthesis, namely IGF-1-induced expression of cyclin D1, E and as a result, pRb phosphorylation.

Few studies have established a link between K+ channel activity and the machinery of the cell cycle. Treatment by pharmacological inhibitors of K+ channels (TEA, 4-AP) failed to modulate the increase in the expression of cyclin D induced by PDGF after 6, 12 and 18 h of treatment, but increased the expression of p27[kip1] and p21[Waf1/Cip1] after 48 h of treatment [10,38]. Similar results were obtained when the membrane potential was depolarized by increasing extracellular K+. The authors proposed that changes in the membrane potential (depolarization) could activate a signaling pathway involving the p27[kip1] and p21[Waf1/Cip1] proteins. Recently, Soriani et al. [39,40] reported an accumulation of cells in G1 phase, an accumulation of the p27[kip1] protein and a reduction in cyclin A expression when they blocked K+ channels. They proposed that the inhibition of K+ channels induced a deterioration
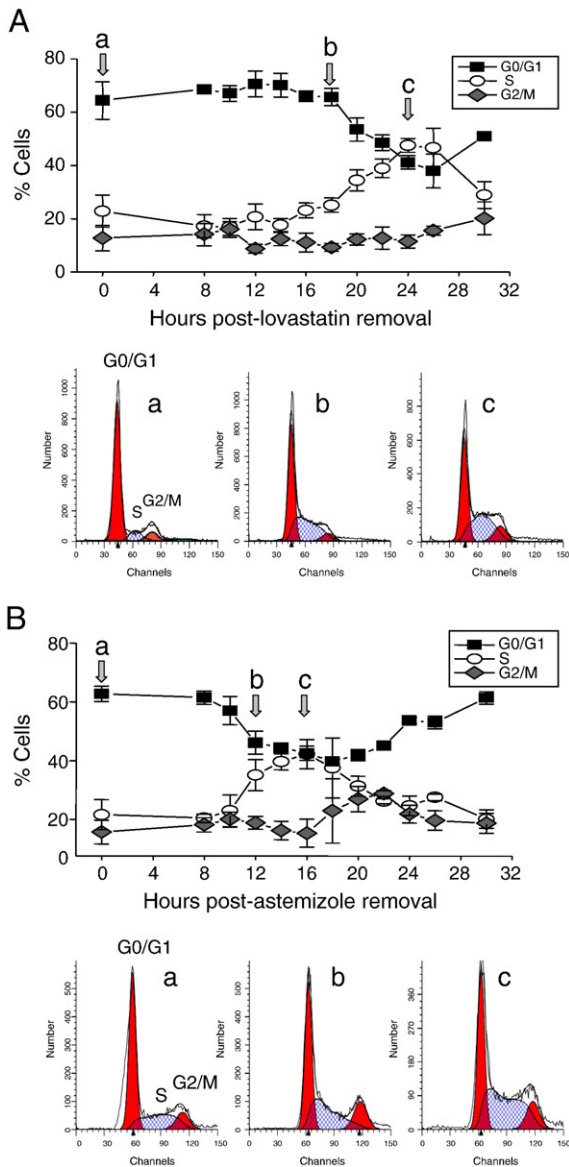
Fig. 5. Temporal position of the arrest site by a hEag1 channel blocker. To determine the temporal position of the arrest site induced by astemizole, we used lovastatin as a control condition for early G1 phase synchronization. MCF-7 cells were thus arrested in G1 phase by either lovastatin (20 μM) (A) or astemizole (5 μM) (B) treatment for 39 h and 48 h, respectively. The medium was replaced with drug free-EMEM medium after astemizole treatment or replaced with drug free-EMEM medium supplemented with 2 mM mevalonic acid after lovastatin treatment (Time = 0). Cells resumed their progression into the cell cycle and were then collected at different times after wash out of these drugs, as indicated. After staining with propidium iodide, the cell distribution in G0/G1, S and G2/M phases was examined by flow cytometry. *Top*, each point represents the mean ± SD from the three experiments. *Bottom*, a representative distribution of the cells in the cell cycle at three defined points of the kinetic (a, b and c).

of the cell volume and a disorganization of the skeleton responsible for the accumulation of the protein p27[kip1]. In our study, the absence of a correlation between the inhibition of hEag1 channels and accumulation of the p27[Kip1] protein suggest that the accumulation of cells in G1 phase is not due to this kind of process. Moreover, Roy et al. [27] reported the non-involvement of the hEag1 channel in cell volume regulation in MCF-7 cells, in contrast to the human ether-a-go-go related channels (HERG), whose involvement has been demonstrated using a specific blocker, E-4031. However, we did not detect any effect on cyclin expression following E-4031 treatment. Finally, it has been proposed that the deterioration of cell volume induced by K+ channel inhibitors could alter the activity and concentration of all cellular compounds involved in proliferative processes

[41]. In the presence of the astemizole treatment, p21[Waf1/Cip1] and CDK expression did not appear to be modified. These results strengthen the hypothesis that hEag1 channels seem to be involved in a more specific pathway regulating cyclin expression.

In MCF-7 cells, the principal effector of cyclin D1 induction by IGF-1 is the Akt protein [22]. The involvement of ion channels in the regulation of Akt phosphorylation has been reported. In fact, the phosphorylation rate of Akt was strongly dependent on the extracellular calcium concentration. A reduction in this concentration to 0.3 mM strongly decreases the phosphorylation of this protein [42,43].

Moreover, it has been well established that the progression of cells through G1 requires calcium events [44,45]. Different calcium actors have been identified as participating in these processes [46,47]. Furthermore, available evidence suggests that Ca^{2+}-dependent pathways are required prior to the activation of cyclin D/CDK4 and pRb hyperphosphorylation [48]. Potassium channel activity might influence mitogenic Ca^{2+} signals by providing and maintaining a sufficient driving force for Ca^{2+} entry [49,50]. In our previous works, we reported that the inhibition of hEag1 channels reduces the concentration of intracellular calcium in synchronized cells in G1 phase [9]. Considered together, we can suggest that the inhibition of hEag1 channels could disturb calcium homeostasis, leading to a reduction of Akt phosphorylation and thus to a decrease of cyclin D1 and E levels induced by IGF-1.

In conclusion, our study brings new elements towards the understanding of the function of hEag1 channels and may lead to the development of ion channel targeted cancer therapy through IGF-1 modulation.

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