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Intermediate Ca^{2+}-Sensitive K^+ Channels are Necessary for Prolactin-Induced Proliferation in Breast Cancer Cells

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Abstract Prolactin (PRL) is a polypeptidic hormone which acts both systemically and locally to cause lactation by interacting with the PRL receptor, a Janus kinase (JAK2)-coupled cytokine receptor family member. Several studies have reported that serum PRL level elevation is associated with an increased risk for breast cancer, and evidence has suggested that PRL is one actor in the pathogenesis and progression of this cancer. We previously reported the involvement of hIKCa1 in breast cell cycle progression and cell proliferation. However, mechanisms by which PRL cooperates with these channels to modulate breast epithelial cell proliferation remain unknown. Our results showed that, in the MCF-7 breast cancer cell line, PRL increased hIKCa1 current density. These channels were functional and regulated the resting membrane potential. The PRL effects were inhibited by TRAM-34 and clotrimazole, the most used hIKCa1 blockers. Moreover, PRL increased proliferation in a dose-dependent manner without overexpressing hIKCa1. To determine whether PRL-induced proliferation and hIKCa1 activity involved the JAK2 pathway, we used pharmacological JAK2 inhibitors (AG490 and JAK inhibitor I). Indeed, PRL-induced JAK2 phosphorylation was required for both cell proliferation and hIKCa1 activity. In the presence of either hIKCa1 blockers or siRNA-hIKCa1, PRL failed to increase cell proliferation and hIKCa1 activity. Taken together, our results demonstrate that PRL plays a role in breast cancer cell proliferation by increasing hIKCa1 activity through the JAK2 signaling pathway.

Keywords MCF-7 cell · K^+ channel · Prolactin · JAK2 pathway · Breast adenocarcinoma · Cell proliferation

Introduction

Prolactin (PRL) is a protein hormone secreted by lactotrophs in the anterior pituitary gland. PRL is known for its roles in mammary gland development. In mammary epithelial cells, where it is secondarily secreted, PRL has been hypothesized to act via an autocrine/paracrine mechanism (Clevenger et al. 1995; Ginsburg and Vonderhaar 1995; Reynolds et al. 1997).

Recent epidemiological studies have shown that PRL is involved in the etiology of breast cancer in premenopausal and postmenopausal women (Tworoger and Hankinson 2008). Indeed, high circulating PRL levels were associated with a 60% increased risk of breast cancer in estrogen receptor- and progesterone receptor-positive tissues as well as in invasive breast tumors (Tworoger et al. 2004; Tworoger and Hankinson 2008; Tworoger et al. 2006).

Multiple studies have reported that exogenous PRL can promote cell proliferation of various mammary tumor epithelial cell lines derived from either mice or humans (Clevenger and Plank 1997; Liby et al. 2003; Schroeder et al. 2002; Vonderhaar 1999). In breast cancer cell lines, including MCF-7, it has been reported that blockade of the PRL receptor (PRLR) using antagonists or anti-PRL

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antibodies prevented PRL-induced cell proliferation (Das and Vonderhaar 1997; Ginsburg and Vonderhaar 1995; Goffin et al. 1996; Llovera et al. 2000). Moreover, it has also been demonstrated that PRL increases proliferation of these cells by facilitating the G_1/S transition, notably by decreasing the $P21^{WAF1}$ level (Schroeder et al. 2002). PRLR is detected in normal and cancerous breast tissue (Gill et al. 2001; Mertani et al. 1998; Reynolds et al. 1997) as well as in breast cancer cell lines (Peirce and Chen 2001). Its expression has been reported to be higher in human mammary tumors compared to normal adjacent tissue (Gill et al. 2001; Reynolds et al. 1997; Touraine et al. 1998) or not strongly upregulated in human breast cancer (Galsgaard et al. 2009).

PRLR activates signals through a complex of kinases including the best-studied pathway JAK2/STAT5 (Bole-Feysot et al. 1998; Goffin et al. 2005; Swaminathan et al. 2008), src and protein kinase C family members, phosphatidylinositol 3-kinase and multiple mitogen-activated protein kinases (MAPKs). The JAK2/STAT5 pathway is involved in MCF-7 cell proliferation through activation of the cyclin D1 gene promoter (Brockman et al. 2002). Moreover, tyrosine phosphorylation is an early crucial step in PRLR signaling that controls K^+ channel activation, calcium influx and/or intracellular calcium mobilization. Indeed, in CHO cells expressing stably the long PRLR isoform, PRL stimulates a 240-pS Ca^{2+}- and voltage-activated K^+ channel through JAK2 phosphorylation (Prevarskaya et al. 1995) and induces an intracellular calcium rise (Sorin et al. 1998). PRL also activated a Ca^{2+}-dependent K^+ current by stimulating calcium influx, probably through a transient receptor potential channel, in the U87-MG cultured human astrocytoma cell line (Ducret et al. 2004). Moreover, in the human prostate cancer cell line LNCaP, it was reported that PRL stimulates the activity of K^+ channels, inducing cell proliferation via tyrosine kinase phosphorylation (Van Coppenolle et al. 2004). However, the role and the identification of K^+ channels in PRL signal transduction have yet to be fully elucidated in breast cancer cells.

Our previous studies have demonstrated the crucial role played by K^+ channels in MCF-7 cell proliferation including hIKCa1 (Ouadid-Ahidouch and Ahidouch 2008). Indeed, hIKCa1 was demonstrated to be involved in the hyperpolarization of the resting membrane potential (RMP), cell proliferation and cell cycle progression. Blockade of hIKCa1 by clotrimazole depolarized the RMP, inhibited cell proliferation and accumulated cells in the G_1 phase (Ouadid-Ahidouch et al. 2004b).

The present study provides the first direct evidence that hIKCa1 channels and JAK2 are the main actors in PRL signal transduction controlling breast cancer cell proliferation.

Materials and Methods

Cell Culture

MCF-7 breast cancer cells from the American Type Culture Collection (Rockville, MD) were routinely grown in Eagle's minimum essential medium (EMEM; Invitrogen, Cergy Pontoise, France) supplemented with 5% fetal bovine serum (FBS; Cambrex, France), 2 mM L-glutamine (Invitrogen), 20 mM HEPES buffer (Invitrogen), 0.219% sodium bicarbonate (Invitrogen) and 100 units/ml penicillin–streptomycin (Invitrogen). Cells were kept at 37°C in a humidified incubator with 5% CO_2, and the cell culture medium was renewed every 48 h.

Cell Count and Viability

MCF-7 cells were grown in 60-mm Petri dishes at a density of 9×10^4 (not transfected) or 12×10^4 (transfected) for 48 h. Then, cells were starved for 6 h and treated with different concentrations of PRL for the dose–response curve or PRL with or without the hIKCa1 blockers or JAK2 inhibitors. After 72 h incubation, MCF-7 growth was assessed using the standard Malassez cell method. Briefly, cells were removed by trypsinization and diluted in trypan blue. Cell counts were performed six times (in a blind manner), and the results were expressed as the percentage of viable cells measured compared to those measured under control conditions.

Electrophysiology

Cells were cultured in 35 mm Petri dishes 48 h before electrophysiological recordings. Experiments were carried out at room temperature using the whole-cell recording mode of the patch-clamp technique with 3–6 MΩ resistance borosilicate fire-polished pipettes (Hirschmann, Laborgerate, Eberstadt, Germany). The cells under investigation were bathed in a standard extracellular solution containing (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 5 glucose and 10 HEPES, adjusted to pH 7.4 with NaOH. For hIKCa1 current measurements, the composition of the internal pipette solution was as follows (in mM): 125 KCl, 2 $MgCl_2$, 10 HEPES, 9.6 $CaCl_2$ and 10 EGTA, adjusted to pH 7.2 with KOH. The calcium and EGTA concentrations in the above solution were adjusted (using WEBMAXC v2.10; http://www.stanford.edu/~cpatton/webmaxc2.htm) to give a free Ca^{2+} concentration of 5 μM. Extracellular and intracellular osmolarities (290–310 mOsm) were measured using the freezing-point depression technique. MCF-7 expresses the large-conductance Ca^{2+}-activated K^+ channels: BK_{Ca} (Ouadid-Ahidouch et al. 2004a). In order to avoid possible contamination by BK_{Ca} current, 2 mM TEA-Cl were added to the extracellular solution. Currents and

membrane potential were recorded under voltage-clamp or current-clamp mode, respectively, with an Axopatch 200 B patch-clamp amplifier (Molecular Devices, Palo Alto, CA). pClamp software (version 8, Molecular Devices) and Labmaster hardware (1322A; Digidata, Broomfield, CO) were used to control voltage and to acquire and analyze data. In all experiments, potential ramps were applied from −100 to +40 mV for 350 ms every 30 s. The holding potential was −40 mV. Electrophysiological data were analyzed using Pclamp software (PClamp version 8, Molecular Devices).

Western Blot

MCF-7 cells were washed with PBS and lysed for 30 min on ice in RIPA buffer (1% Triton X-100, 1% Na deoxycholate, 150 mM NaCl, 10 mM PO_4Na_2/K [pH 7.2]) supplemented with protease inhibitor cocktail (P8340; Sigma, Saint-Quentin Fallavier, France), 2 mM EDTA and 5 mM Na orthovanadate. After centrifugation (12,000×*g*, 5 min, 4°C), proteins in supernatant were quantified using the BCA method (Bio-Rad, Marnes-la-Coquette, France); equal amounts of each protein sample (40 µg) were then loaded onto a 5% stacking/8% running SDS–polyacrylamide gel and separated by electrophoresis. After transfer to nitrocellulose membrane (2 h, 60 V, Hybond-C extra; Amersham, Pantin, France), membranes were blocked in 5% fat-free milk for 90 min at room temperature in TBS-Tween (Tris 20 mM [pH 6.7], NaCl 150 mM, Tween 0.1%). Blots were then incubated overnight at 4°C with primary antibodies raised against hIKCa1 (1/400, clone H-120), p-JAK2 (1/400, sc-21870), JAK2 (1/400, clone C-20) and *β*-actin (1/1,000, clone c-11; all from Santa Cruz Biotechnology, Heidelberg, Germany). Detection was performed for 1 h at room temperature using peroxidase-conjugated anti-goat or rabbit IgG secondary antibodies (1/10,000 and 1/5,000, respectively) and an enhanced chemiluminescence system (Amersham and Bio-Rad). Densitometric analysis of bands was quantitated using the Chemidoc system and Quantity One software (Bio-Rad).

siRNA Cell Transfection

MCF-7 cells were transiently transfected using nucleofection technology according to the Amaxa Biosystems (Cologne, Germany) protocol. Briefly, 2×10^6 cells were resuspended in 100 µl of Cell Line Nucleofector Solution V; then, the cell suspension was mixed with 2 µg of siRNA (1.3 µM for 2 million cells) directed against hIKCa1 (sense 5′-UGUAAAGCUUGGCCACGAAC-3′, antisense 5′-GUUCGUGGCCAAGCUUUACA-3′; Dharmacon, Lafayette, CO) or siRNA control (siGENOME Non-Targeting siRNA, Dharmacon). The sample was transferred into an electroporation cuvette, and transfection was performed using the program E-14 according to the manufacturer's instructions. Immediately after nucleofection, cells were transferred into prewarmed complete maintenance medium and cultured as described above.

Chemicals

PRL (Sigma, France) was reconstituted in 4 mM HCl solution containing 1 mg/ml bovine serum albumin. TRAM-34, clotrimazole, JAK inhibitor I and AG490 (Sigma, France) were used in DMSO. Final concentrations were obtained by appropriate dilution in an external control solution. The final DMSO concentration was <0.1%.

Statistical Analysis

Data are presented as mean ± SEM. Statistical significance was determined using Student's *t* test or ANOVA (SigmaStat; Systat Software, San Jose, CA) with Holm-sidak post hoc analysis. $P < 0.05$ was considered significant.

Results

Expression of Functional hIKCa1 Channels in MCF-7 Cells

In order to isolate hIKCa1 current, we used two different protocols. We dialyzed the cytoplasm during whole-cell recording (1) with 5 µM free Ca^{2+} pipette solution or (2) with a standard pipette solution and extracellular perfusion of 1 µM ionomycin. The activity of hIKCa1 channels was then blocked by the most used hIKCa1 channel blockers (clotrimazole and TRAM-34). Ramp currents were elicited by 350-ms voltage ramps from −100 to +40 mV (Fig. 1A–D, insets). Both 1 µM ionomycin and 5 µM free Ca^{2+} pipette solution activated a linear current that was greatly reduced by 5 µM clotrimazole (CLT, $n = 10$; Fig. 1A, C) and 10 µM TRAM-34 ($n = 5$; Fig. 1B, D). The clotrimazole (I_{Clo})- and TRAM-34 ($I_{TRAM\text{-}34}$)-subtracted current densities were not significantly different ($I_{Clo} = 18.7 \pm 2.2$ pA/pF [$n = 10$] and $I_{TRAM\text{-}34} = 12.1 \pm 4.0$ pA/pF [$n = 5$], both measured at +40 mV). Moreover, in both cases, the reversal potential was around −80 mV. The same reversal potential value was found using either 1 µM ionomycin (Fig. 1A, C, insets) or 5 µM free Ca^{2+} pipette solution (Fig. 1B, D, insets). This result is close to the calculated value of the Nernst potential for potassium ($E_K = -81$ mV), supporting a K^+-selective conductance. I_{Clo} and $I_{TRAM\text{-}34}$ currents presented a reversal potential at -83.4 ± 0.07 mV ($n = 10$) and -80.6 ± 0.06 mV ($n = 5$), respectively (Fig. 1E, F).

We also investigated the role of hIKCa1 in regulating the RMP. The RMP measured using 5 μM free Ca^{2+} pipette solution was around −50 mV (−47.1 ± 8.6 mV, $n = 18$) and showed a large depolarization when perfusing either 5 μM clotrimazole (−27.7 ± 4.3, $n = 10$) or 10 μM TRAM-34 (−33 ± 7.9, $n = 8$).

To confirm that hIKCa1 K^+ channels are functional in MCF-7 cells, we used siRNA technology to downregulate hIKCa1. Cells were transfected with siRNA-hIKCa1 or with nontargeted siRNA (siCtrl). Compared to control at 72 h posttransfection, sihIKCa1-transfected cells showed a protein level decrease by 41 ± 9% (Fig. 2A). In sihIKCa1-transfected cells, the K^+ current density (measured at +40 mV) activated by 5 μM Ca^{2+} pipette was reduced to about 70% of the control ($P < 0.001$, $n = 12$, Fig. 2B). Taken together, these results provided direct evidence that functional hIKCa1 channels are expressed in MCF-7 cells.

PRL Increased hIKCa1 Current

To determine if there is a relationship between PRL and K^+ channel activity, we used the whole-cell configuration of the patch-clamp technique. K^+ current was elicited by voltage ramps from −120 to +40 mV from a holding potential of −40 mV. MCF-7 cell exposure to PRL (100 ng/ml) led to an increase in the amplitude of the K^+ current density (Fig. 3A [a, b]). The time course showed that the amplitude of the K^+ current density increased by 50 ± 7.5% ($n = 15$) within 4.8 ± 1 min after exposure to PRL. Furthermore, the PRL-subtracted current showed an E_{rev} of about −80 mV (−79.5 ± 2.3 mV, $n = 10$, Fig. 3A [c]), attesting to the K^+ selectivity of the activated channels. We then investigated whether PRL stimulated hIKCa1 channel activity. We found that both hIKCa1 blockers (TRAM-34 and clotrimazole) completely inhibited the PRL-induced K^+ current increase (Fig. 3B, C). The effects of both 10 μM TRAM-34 and 5 μM clotrimazole were reversible (Fig. 3B, C). Moreover, in siCTRL-transfected cells, PRL increased the current density ($P < 0.05$, $n = 8$; Fig. 3D) but failed to increase that of the sihIKCa1-transfected cells (Fig. 3D). Furthermore, the slight hyperpolarization of the RMP induced by PRL as well as that induced by 5 μM intracellular free Ca^{2+} was completely suppressed in the presence of either TRAM-34 or clotrimazole

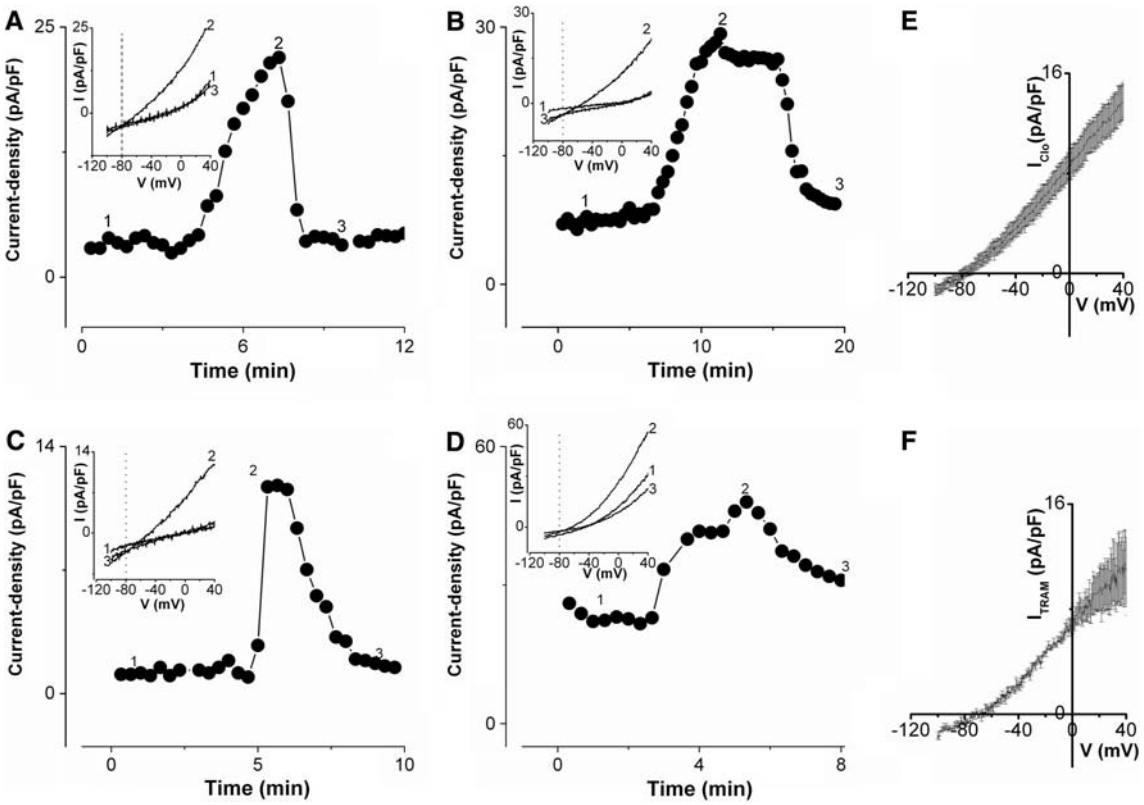

Fig. 1 hIKCa1 channels are functional in MCF-7 cells. **A–D** Time-course activation of Ca^{2+}-sensitive K^+ current measured at +40 mV. The K^+ current, elicited with extracellular perfusion of 1 μM ionomycin (**A**, **C** 1; Ctrl 2) or with 5 μM free Ca^{2+} pipette (**B**, **D** 1; Ctrl 2), was blocked by 5 μM clotrimazole (**A**, **B** 3) and 10 μM TRAM-34 (**C**, **D** 3); representative traces are shown as *insets*. **E**, **F** Mean hIKCa1 current density/voltage relationship sensitive to clotrimazole (**E** I_{CLO}, $n = 10$) or TRAM-34 (**F** I_{TRAM}, $n = 5$)

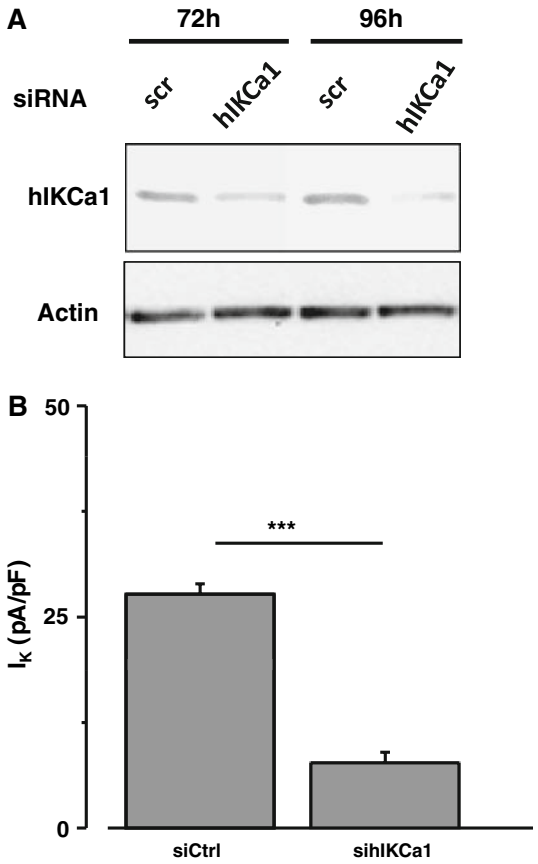

Fig. 2 siRNA directed against hIKCa1 abolished hIKCa1 activity. **A** MCF-7 cells were transfected with siRNA scramble (scr, siCtrl) or sihIKCa1 and cultured for 72 and 96 h. Western blot gel is representative of three independent experiments. **B** Mean K^+ current densities elicited with 5 μM Ca^{2+} pipette at +40 mV in MCF-7 cells transfected with siCtrl and sihIKCa1 (*** $P < 0.001$ vs. control)

(Fig. 3E). Similar results were obtained when we transfected cells with sihIKCa1. Indeed, in siCTRL-transfected cells, PRL induced hyperpolarization of the RMP (-55.8 ± 1.64 mV, $n = 13$; Fig. 3F). In contrast, in sihIKCa1, the RMP was depolarized (-13.5 ± 1.9 mV, $n = 13$) and PRL failed to induce hyperpolarization ($n = 13$, Fig. 3F).

PRL Increased Cell Proliferation Without Overexpression of hIKCa1 Channels

To evaluate the mitogenic effect of PRL; cells were incubated in serum-deprived medium for 6 h and treated with PRL for 72 h. Figure 4A shows that PRL increased cell proliferation in a dose-dependent manner. PRL induced a proliferative effect at 50 ng/ml ($13.4 \pm 4.4\%$), and the maximal increase was obtained for 100 or 150 ng/ml ($20.2 \pm 3.9\%$ and $28.3 \pm 4.7\%$), respectively. We also investigated whether PRL induced cell mortality under our experimental conditions. For each condition (with or without PRL), the percentage of dead cells was <5% (data not shown). To determine whether PRL-induced proliferation was correlated to the variation in hIKCa1 expression, we examined the effect of PRL on hIKCa1 protein levels. Densitometric analysis of Western blots showed that PRL dose- and time-dependent treatment did not alter hIKCa1 levels (Fig. 4B, C).

JAK2 Is Involved in PRL-Dependent Proliferation and hIKCa1 K^+ Channel Activity

To determine whether PRL-induced cell proliferation through JAK2 phosphorylation, we used two specific chemical inhibitors of JAK2 phosphorylation: AG 490 and JAK inhibitor I. Figure 5A shows that PRL induced JAK2 phosphorylation in a time-dependent manner. Phosphorylation of JAK2 was observed after 2.5 min exposure to PRL, and the maximum effect was observed at 20 min (Fig. 5A). Preincubation with 0.17 μM of AG 490 and 15 nM of JAK inhibitor I for 10 and 20 min totally abolished PRL-stimulated JAK2 phosphorylation (Fig. 5B). We also investigated the involvement of JAK2 in PRL-induced cell proliferation. Figure 5C shows that 150 ng/ml PRL increased significantly cell proliferation ($35.9 \pm 2.3\%$). This effect was reduced to $11.4 \pm 3.1\%$ by 0.17 μM AG 490 and completely abolished by 15 nM JAK inhibitor I (Fig. 5C). Furthermore, PRL failed to increase cell proliferation in the presence of 1 μM herbimycin A, the well-known tyrosine kinases inhibitor (Fig. 5C). None of the three tyrosine kinase blocker affected cell proliferation when applied alone in control conditions (serum-free medium, Fig. 5C). In addition, we investigated the effect of AG 490 and JAK inhibitor I on hIKCa1 activity. Cells were pretreated with AG 490 (1 μM) and JAK inhibitor I (0.2 μM) for 30 min before recording hIKCa1 activity. Both AG 490 and JAK inhibitor I completely suppressed the PRL-induced increase in K^+ current density ($n = 12$, Fig. 5D, E). Taken together, these results suggest that JAK2 participates directly in the PRL-induced hIKCa1 channel activity and cell proliferation.

PRL Failed to Activate Cell Proliferation in the Presence of hIKCa1 Blockers or siRNA Against hIKCa1

Pharmacological inhibition of hIKCa1 channel activity by 5 μM TRAM-34 or by 5 μM clotrimazole largely attenuated PRL-induced mitogenesis in MCF-7 cells, while 2 mM TEA failed to decrease the PRL effect on cell proliferation (Fig. 6A). Furthermore, when used alone, all these drugs had no effect on cell proliferation in serum-free medium (Fig. 6A). To further support and specify the importance of hIKCa1 channels in the PRL mitogenic

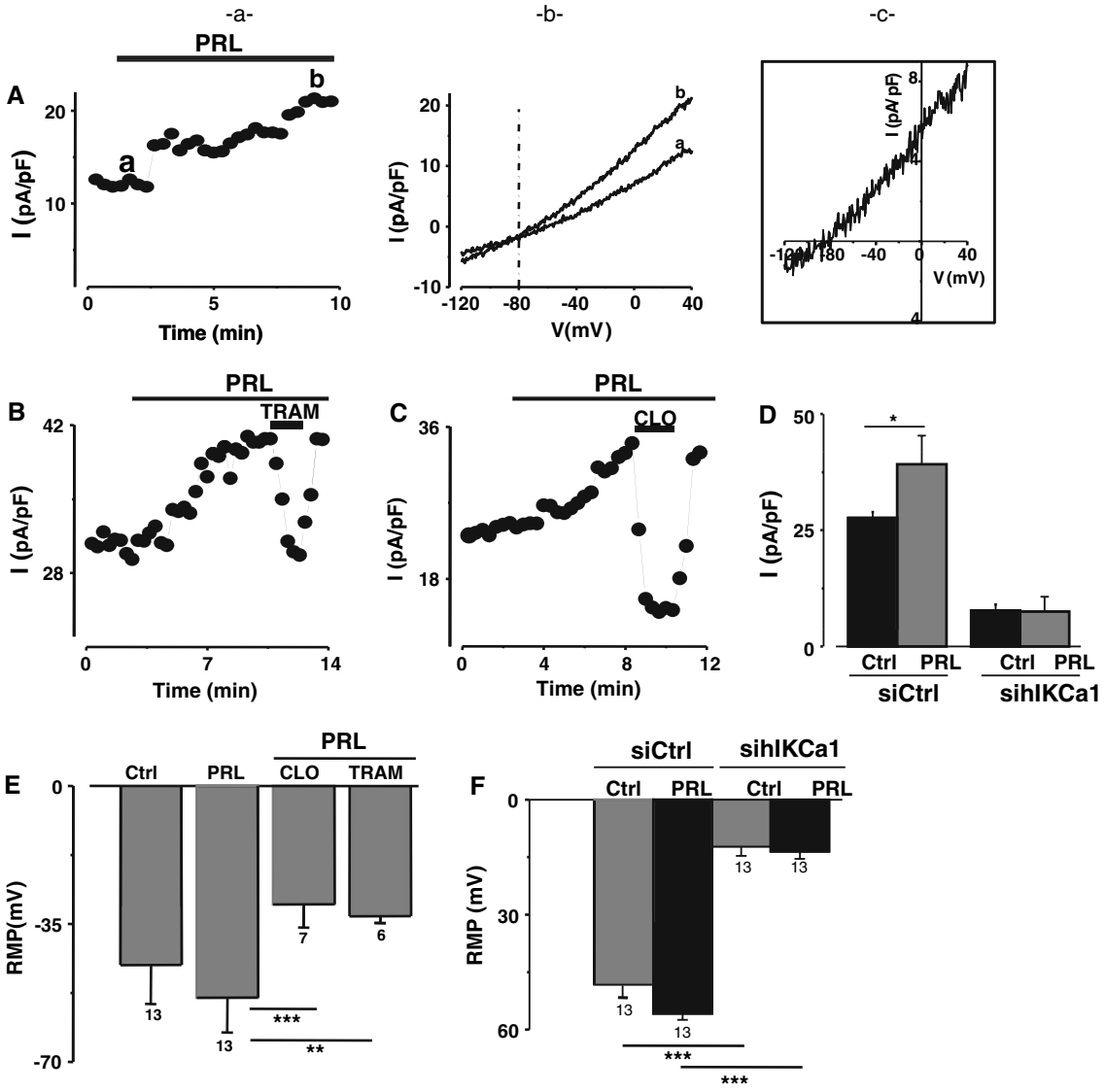

Fig. 3 PRL effect on hIKCa1 current. **A** (*a*) Time course of 100 ng/ml PRL on K^+ current measured at +40 mV. **A** (*b*) Current–density traces recorded under control conditions (*a*) and after perfusion of PRL (*b*). **A** (*c*) PRL-sensitive K^+ current obtained by subtraction of the two traces (*b* and *a*). Inhibition of the K^+ current stimulated by 100 ng/ml PRL by 10 μM TRAM-34 (TRAM, **B**), 5 μM clotrimazole (CLO, **C**) or sihIKCa1 (**D**). **E**, **F** Mean RMP under control conditions, PRL condition and after application of CLO, TRAM (**E**) or sihIKCa1 (**F**). ** $P < 0.01$, *** $P < 0.001$ vs. control

effect in MCF-7 cells, we used siRNA technology to downregulate hIKCa1 expression. Cells were transfected with siRNA-hIKCa1 or with nontargeted siRNA (siCtrl). Following siRNA transfection according to the Amaxa Biosystems protocol, cells were further cultured in medium containing 5% serum for 48 h and starved for 6 h, then PRL was added at 150 ng/ml for 72 h. PRL 150 ng/ml increased cell proliferation by 30.1 ± 2.1% and 31.4 ± 1% in electroporated cells (without siRNA) and siCtrl transfected cells, respectively (Fig. 6B). In contrast, it failed to increase MCF-7 cell proliferation in cells transfected with sihIKCa1 (Fig. 6B). Taken together, these results demonstrate that hIKCa1 channels are involved in MCF-7 cell proliferation induced by PRL.

Discussion

Several studies have reported that PRL increases cell proliferation in breast cell lines (Biswas and Vonderhaar 1987; Das and Vonderhaar 1997; Llovera et al. 2000). Moreover, our previous study showed the involvement of K^+ channels in MCF-7 proliferation (Ouadid-Ahidouch and Ahidouch 2008). Although the transduction pathways involved in the

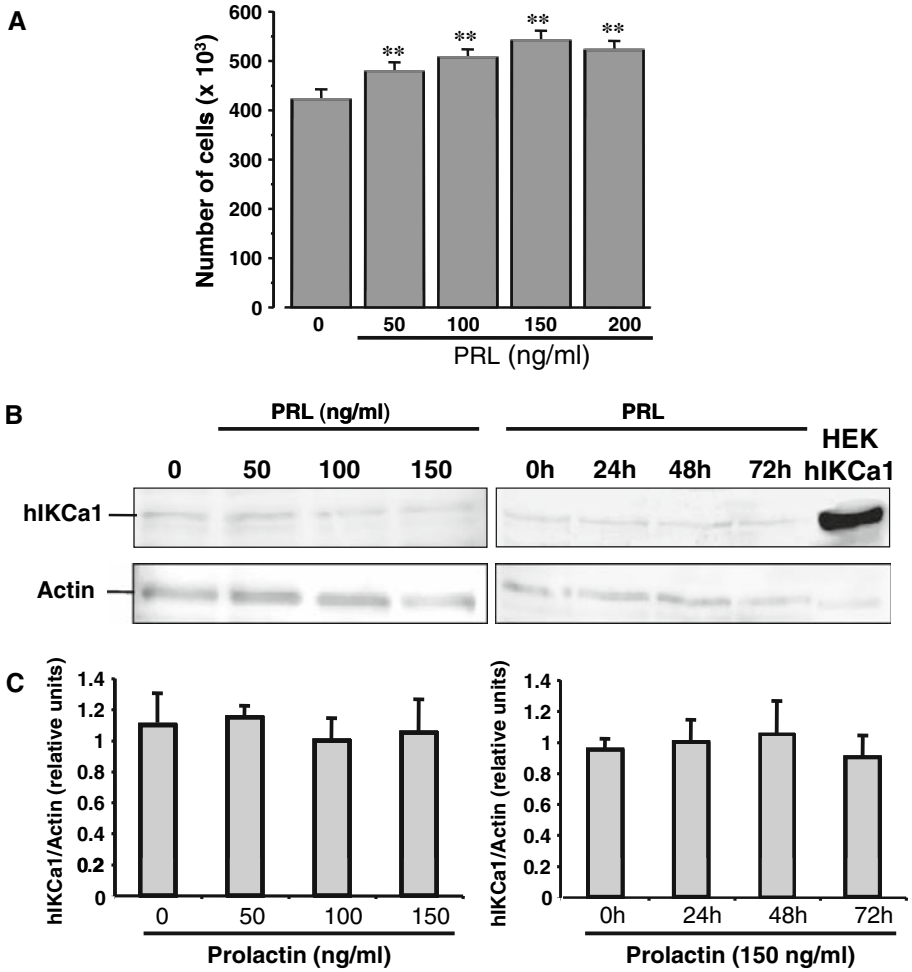

Fig. 4 PRL increased MCF-7 cell proliferation without hIKCa1 overexpression. **A** Cells were serum- and phenol red-deprived for 6 h and treated with various concentrations of PRL for 72 h. Mean of three independent experiments (** $P < 0.01$). **B** Western blot of hIKCa1 protein from lysates of MCF-7 cells treated with various concentrations of PRL for 72 h (*left panel*) or with 150 ng/ml PRL for 24, 48 and 72 h (*right panel*). HEK-hIKCa1 cells were used as positive control. Western blots are representatives of three independent experiments. **C** hIKCa1 quantitation (bar graph) was performed using densitometry. Data were normalized to β-actin. Graph and Western blot are representative of three independent experiments, respectively

proliferative PRL effect in MCF-7 cells have been studied in great detail, very little is known about a PRL effect on the K^+ conductance in these cells. In the present study, we show that (1) hIKCa1 channels are functional and regulate the membrane potential of MCF-7 cells; (2) PRL increases hIKCa1 channel activity, probably through JAK2 tyrosine kinase; and (3) hIKCa1 channels are involved in the growth-stimulatory effect of PRL through the JAK2 pathway. We can thus suggest that hIKCa1 K^+ channels constitute a target of PRL, leading to breast cancer cell proliferation.

We previously reported that hIKCa1 current density varies during the cell cycle and contributes to the progression through the G_1 phase and G_1/S transition by controlling the RMP (Ouadid-Ahidouch et al. 2004b). Here, our electrophysiological results confirm the functionality of hIKCa1 and its contribution to the regulation of the membrane potential. Indeed, either ionomycin or 5 μM free intracellular Ca^{2+} activated a linear current with an E_{rev} of −80 mV and induced hyperpolarization. Perfusion of the two known hIKCa1 blockers (TRAM-34 and clotrimazole) inhibited the linear current and depolarized the membrane potential.

The early experiments carried out by Duffy's group, which intensively studied the PRL signaling pathways on ion channels using CHO cells stably transfected with the cDNA of the long form of rabbit mammary PRLR and the human malignant astrocytoma U87-MG cell line, showed that PRL induced membrane hyperpolarization, caused by Ca^{2+}-activated K^+ channel stimulation (Ducret et al. 2004; Prevarskaya et al. 1994; Vacher et al. 1994). In our conditions, PRL increased the linear current activated either by ionomycin or by 5 μM free Ca^{2+} pipette solution. Both TRAM-34 and clotrimazole completely suppressed the PRL-induced current and depolarized the membrane potential. The time course of the PRL effect requires several minutes. Indeed, the maximum effect was observed at 5 min after application of PRL and was irreversible even after 30 min of continuous washout. The effect of PRL on K^+ current could be due to a direct/indirect effect of the K^+ channels or an increase in $[Ca^{2+}]_i$. PRL induced an increase in $[Ca^{2+}]_i$ in Nb2 lymphoma (Ali et al. 1991), hepatocytes of lactating rats, INS-1 insulinoma (Sekine et al. 1996), CHO stably expressed PRLR (Ratovondrahona et al. 1998) and U87-MG cells (Ducret et al. 2002). In

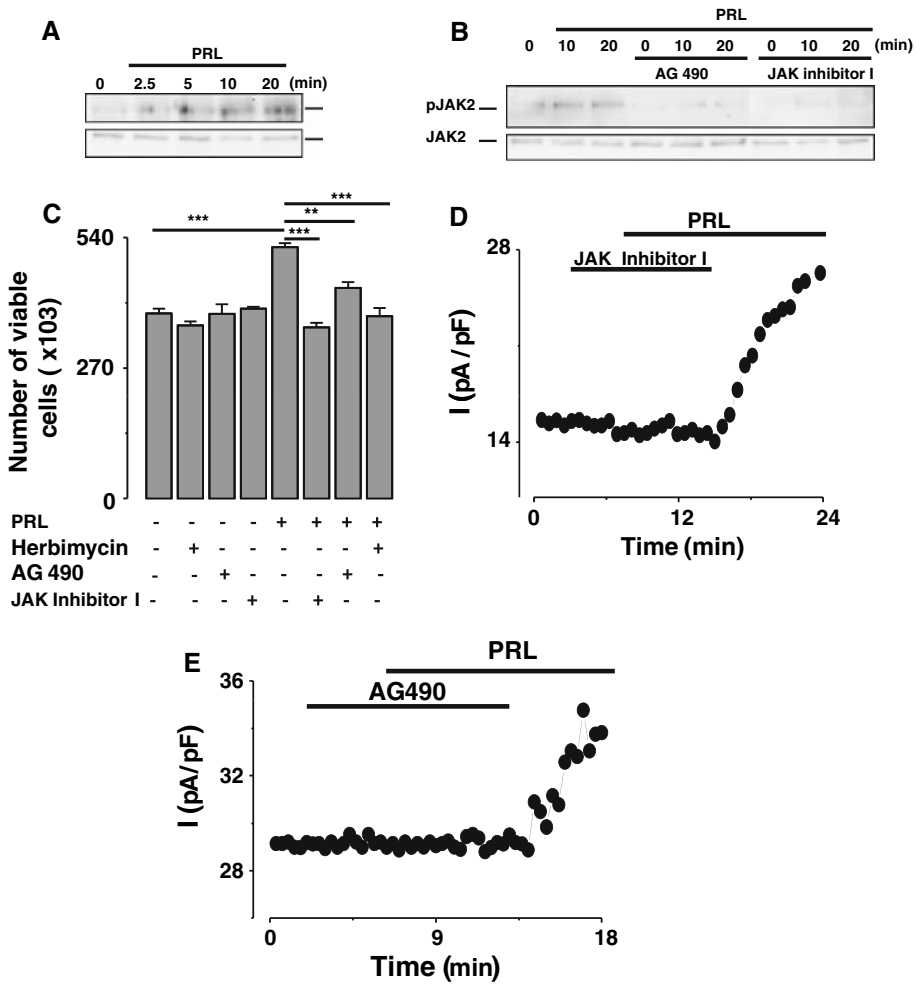

Fig. 5 PRL induced MCF-7 cell proliferation and hIKCa1 activation via JAK2 phosphorylation. **A** Western blot of pJAK2 and JAK2 proteins realized from lysates of MCF-7 cells. Cells were serum- and phenol red-deprived for 6 h and then incubated with PRL (150 ng/ml) for 2.5–20 min. **B** MCF-7 treatment with AG 490 (0.17 μM) and JAK inhibitor I (15 nM) 12 h before stimulation with PRL (150 ng/ml) for 10–20 min at 37°C. **C** Cells were serum- and phenol red-deprived for 6 h and treated in the absence or presence of PRL (150 ng/ml), herbimycin (1 μM), AG 490 (0.17 μM) and JAK inhibitor I (15 nM) for 72 h. Graph or Western blots are representative of three independent experiments (** $P < 0.01$, *** $P < 0.001$). Cell pretreatment with AG 490 or JAK inhibitor I completely suppressed the PRL-induced increase in K^+ current density. **D**, **E** Representative time-course curves of the effect of JAK inhibitor I and AG 490 on the PRL response on K^+ current, respectively

MCF-7 cells, despite numerous studies on PRL effects, only one study reported a PRL effect on Ca^{2+} homeostasis. Indeed, addition of PRL (1 μg/ml) to MCF-7 cells did not affect the cytosolic Ca^{2+} concentration (Anantamongkol et al. 2007). In our conditions, we showed that PRL was unable to increase K^+ current when hIKCa1 is not activated, suggesting that intracellular Ca^{2+} is not involved in PRL signal transduction in MCF-7 cells.

Tyrosine kinase regulation is obviously an essential actor in PRL signal transduction (Clevenger et al. 2003). Our immunoblotting experiments showed that PRL was able to phosphorylate JAK2. Moreover, both JAK inhibitor I and AG-490 inhibited this phosphorylation. Furthermore, using these inhibitors, our electrophysiological studies showed that this kinase regulated hIKCa1 channel activity. PRL has been reported to increase voltage-activated K^+ channel activity through tyrosine kinase phosphorylation in human cancer prostate cells (Van Coppenolle et al. 2004). It was also reported that PRL increased Ca^{2+}-activated K^+ channel activity via JAK2. Indeed, PRL was unable to activate K^+ current in the presence of an anti-JAK2 antibody in the patch pipette (Prevarskaya et al. 1995). In agreement with these studies and based on the observed inhibitory effects of JAK2 inhibitors on both JAK2 phosphorylation and hIKCa1 channel activity induced by PRL in MCF-7, we proposed that JAK2 activity is involved in the positive regulation of this channel activity, probably by phosphorylation.

hIKCa1 has been reported to control cell proliferation in several cancer cell types, such as prostate (Parihar et al. 2003), pancreatic (Jager et al. 2004), endometrial (Wang et al. 2007) and MCF-7 (Ouadid-Ahidouch et al. 2004b) cells. Recently, we reported a positive correlation between hIKCa1 expression and tumor grade (Haren et al. 2010). Indeed, hIKCa1 mRNA and protein are highly expressed in grade III tumors compared to grades I and II. Moreover, hIKCa1 channels are expressed in epithelial breast cancer cells and are functional (Haren et al. 2010).

Our results showed that PRL increased cell proliferation in a dose-dependent manner and treatment of MCF-7 by hIKCa1 channel pharmacological inhibitors or by si-hIKCa1 inhibited the PRL mitogenic effect. Moreover,

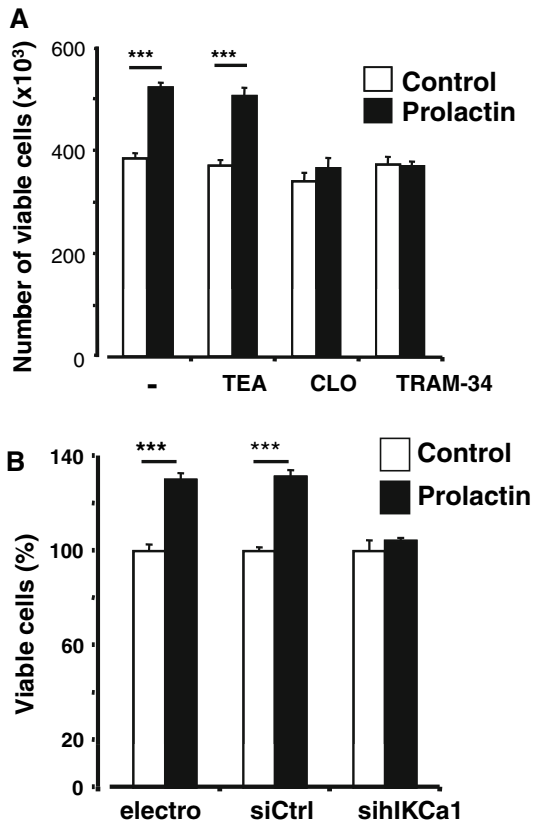

Fig. 6 siRNA against hIKCa1 abolished the mitogenic effect induced by PRL. **A** MCF-7 cells were treated in the absence or presence of PRL (150 ng/ml), TEA (2 mM), CLO (5 μM) and TRAM-34 (5 μM) for 72 h (*** $P < 0.001$). **B** MCF-7 cells were transfected with siCtrl or sihIKCa1, then serum- and phenol red-deprived for 6 h. Starved cells were treated with PRL (150 ng/ml) for 72 h. Each graph is representative of three independent experiments (*** $P < 0.001$)

hIKCa1 siRNA treatment drastically decreased the K^+ current amplitude activated by 5 μM free Ca^{2+} pipette solution and prevented the PRL effect. This finding is associated with a depolarized membrane. Taken together, our results indicated that hIKCa1 channels are important for cell proliferation induced by PRL. To our knowledge, only one study has demonstrated involvement of K^+ channels in the PRL proliferative effect, in LNCaP human prostate cancer cells (Van Coppenolle et al. 2004).

In conclusion, the present report demonstrates that PRL stimulated the activity of the hIKCa1 channels that are involved in cell proliferation via JAK2 in MCF-7 cells. We suggested that PRL activated K^+ channels, not via their overexpression, but through JAK2 phosphorylation, leading to cell proliferation.

As the PRLRs are overexpressed in human mammary tumors (Gill et al. 2001; Touraine et al. 1998) and hIKCa1 channels are also expressed in these tissues (Haren et al. 2010), the link we have established here between PRL-induced proliferation and hIKCa1 channels provides a clue for understanding their participation in the development of breast cancer.

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